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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification n 6:

C07K 14/00, A01N 37/18, 43/04, C07H
17/00, C12N 5/00, C12P 21/06

A1

(11) International Publication Number:

WO 96/27610

(43) International Publication Date: 12 September 1996 (12.09.96)

(21) International Application Number: PCT/US96/03172

(22) International Filing Date: 7 March 1996 (07.03.96)

(30) Priority Data:

400,159

7 March 1995 (07.03.95)

US

(60) Parent Application or Grant

(63) Related by Continuation

US

Filed on

400,159 (CIP)

7 March 1995 (07.03.95)

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(81) Designated States: AL, AM, AU, AZ, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, IS, JP, KG, KP, KR, KZ, LK, LR, LS, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, TR, TT, UA, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: NUCLEOTIDE AND PROTEIN SEQUENCES OF VERTEBRATE SERRATE GENES AND METHODS BASED THEREON

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NUCLEOTIDE AND PROTEIN SEQUENCES OF
VERTEBRATE SERRATE GENES AND METHODS BASED THEREON

This invention was made in part with government support under Grant numbers GM 29093 and NS 26084 awarded by the Department of Health and Human Services. The government has certain rights in the invention.

1. INTRODUCTION

The present invention relates to vertebrate Serrate genes and their encoded protein products, as well as derivatives and analogs thereof. Production of vertebrate Serrate proteins, derivatives, and antibodies is also provided. The invention further relates to therapeutic compositions and methods of diagnosis and therapy.

2. BACKGROUND OF THE INVENTION

Genetic analyses in *Drosophila* have been extremely useful in dissecting the complexity of developmental pathways and identifying interacting loci. However, understanding the precise nature of the processes that underlie genetic interactions requires a knowledge of the protein products of the genes in question.

Embryological, genetic and molecular evidence indicates that the early steps of ectodermal differentiation in *Drosophila* depend on cell interactions (Doe and Goodman, 1985, Dev. Biol. 111:206-219; Technau and Campos-Ortega, 1986, Dev. Biol. 195:445-454; Vassin et al., 1985, J. Neurogenet. 2:291-308; de la Concha et al., 1988, Genetics 118:499-508; Xu et al., 1990, Genes Dev. 4:464-475; Artavanis-Tsakonas, 1988, Trends Genet. 4:95-100). Mutational analyses reveal a small group of zygotically-acting genes, the so called neurogenic loci, which affect the choice of ectodermal cells between epidermal and neural pathways (Poulson, 1937, Proc. Natl. Acad. Sci. 23:133-137; Lehmann et al., 1983, Wilhelm Roux's Arch. Dev. Biol. 192:62-74; Jürgens et al., 1984, Wilhelm Roux's Arch. Dev. Biol.

193:283-295; Wieschaus et al., 1984, Wilhelm Roux's Arch. Dev. Biol. 193:296-307; Nüsslein-Volhard et al., 1984, Wilhelm Roux's Arch. Dev. Biol. 193:267-282). Null mutations in any one of the zygotic neurogenic loci -- *Notch* (*N*), *Delta* (*Dl*), *mastermind* (*mam*), *Enhancer of Split* (*E(spl)*), *neuralized* (*neu*), and *big brain* (*bib*) -- result in hypertrophy of the nervous system at the expense of ventral and lateral epidermal structures. This effect is due to the misrouting of epidermal precursor cells into a neuronal pathway, and implies that neurogenic gene function is necessary to divert cells within the neurogenic region from a neuronal fate to an epithelial fate. *Serrate* has been identified as a genetic unit capable of interacting with the *Notch* locus (Xu et al., 1990, Genes Dev. 4:464-475). These genetic and developmental observations have led to the hypothesis that the protein products of the neurogenic loci function as components of a cellular interaction mechanism necessary for proper epidermal development (Artavanis-Tsakonas, S., 1988, Trends Genet. 4:95-100).

Mutational analyses also reveal that the action of the neurogenic genes is pleiotropic and is not limited solely to embryogenesis. For example, ommatidial, bristle and wing formation, which are known also to depend upon cell interactions, are affected by neurogenic mutations (Morgan et al., 1925, Bibliogr. Genet. 2:1-226; Welshons, 1956, Dros. Inf. Serv. 30:157-158; Preiss et al., 1988, EMBO J. 7:3917-3927; Shellenbarger and Mohler, 1978, Dev. Biol. 62:432-446; Technau and Campos-Ortega, 1986, Wilhelm Roux's Arch. Dev. Biol. 195:445-454; Tomlison and Ready, 1987, Dev. Biol. 120:366-376; Cagan and Ready, 1989, Genes Dev. 3:1099-1112).

Sequence analyses (Wharton et al., 1985, Cell 43:567-581; Kidd and Young, 1986, Mol. Cell. Biol. 6:3094-3108; Vässin, et al., 1987, EMBO J. 6:3431-3440; Kopczynski, et al., 1988, Genes Dev. 2:1723-1735) have shown that two of the neurogenic loci, *Notch* and *Delta*, appear to encode transmembrane proteins that span the membrane a single time. The *Notch* gene encodes a ~300 kd protein (we use "Notch" to

denote this protein) with a large N-terminal extracellular domain that includes 36 epidermal growth factor (EGF)-like tandem repeats followed by three other cysteine-rich repeats, designated *Notch/lin-12* repeats (Wharton, et al., 1985, Cell 5 43:567-581; Kidd and Young, 1986, Mol. Cell. Biol. 6:3094-3108; Yochem, et al., 1988, Nature 335:547-550). *Delta* encodes a ~100 kd protein (we use "Delta" to denote DLZM, the protein product of the predominant zygotic and maternal transcripts; Kopczynski, et al., 1988, Genes Dev. 2:1723-10 1735) that has nine EGF-like repeats within its extracellular domain (Vässin, et al., 1987, EMBO J. 6:3431-3440; Kopczynski, et al., 1988, Genes Dev. 2:1723-1735). Molecular studies have lead to the suggestion that Notch and Delta constitute biochemically interacting elements of a cell 15 communication mechanism involved in early developmental decisions (Fehon et al., 1990, Cell 61:523-534).

The EGF-like motif has been found in a variety of proteins, including those involved in the blood clotting cascade (Furie and Furie, 1988, Cell 53: 505-518). In 20 particular, this motif has been found in extracellular proteins such as the blood clotting factors IX and X (Rees et al., 1988, EMBO J. 7:2053-2061; Furie and Furie, 1988, Cell 53: 505-518), in other *Drosophila* genes (Knust et al., 1987 EMBO J. 761-766; Rothberg et al., 1988, Cell 55:1047-1059), 25 and in some cell-surface receptor proteins, such as thrombomodulin (Suzuki et al., 1987, EMBO J. 6:1891-1897) and LDL receptor (Sudhof et al., 1985, Science 228:815-822). A protein binding site has been mapped to the EGF repeat domain in thrombomodulin and urokinase (Kurosawa et al., 1988, J. 30 Biol. Chem 263:5993-5996; Appella et al., 1987, J. Biol. Chem. 262:4437-4440). The *Drosophila* *Serrate* gene has been cloned and characterized (PCT Publication WO 93/12141 dated June 24, 1993). However, prior to the present invention, despite attempts to achieve the same, no vertebrate *Serrate* 35 gene was available.

Citation of references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

5

3. SUMMARY OF THE INVENTION

The present invention relates to nucleotide sequences of vertebrate Serrate genes (human Serrate and related genes of other species), and amino acid sequences of their encoded proteins, as well as derivatives (e.g.,
10 fragments) and analogs thereof. Nucleic acids hybridizable to or complementary to the foregoing nucleotide sequences are also provided. In a specific embodiment, the Serrate protein is a human protein.

The invention relates to vertebrate Serrate
15 derivatives and analogs of the invention which are functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length (wild-type) Serrate protein. Such functional activities include but are not limited to antigenicity
20 [ability to bind (or compete with Serrate for binding) to an anti-Serrate antibody], immunogenicity (ability to generate antibody which binds to Serrate), ability to bind (or compete with Serrate for binding) to Notch or other toporythmic proteins or fragments thereof ("adhesiveness"), ability to
25 bind (or compete with Serrate for binding) to a receptor for Serrate. "Toporythmic proteins" as used herein, refers to the protein products of *Notch*, *Delta*, *Serrate*, *Enhancer of split*, and *Deltex*, as well as other members of this interacting gene family which may be identified, e.g., by
30 virtue of the ability of their gene sequences to hybridize, or their homology to *Delta*, *Serrate*, or *Notch*, or the ability of their genes to display phenotypic interactions.

The invention further relates to fragments (and derivatives and analogs thereof) of vertebrate Serrate which
35 comprise one or more domains of the Serrate protein, including but not limited to the intracellular domain, extracellular domain, transmembrane domain, membrane-

associated region, or one or more EGF-like (homologous) repeats of a Serrate protein, or any combination of the foregoing.

Antibodies to vertebrate Serrate, its derivatives
5 and analogs, are additionally provided.

Methods of production of the vertebrate Serrate proteins, derivatives and analogs, e.g., by recombinant means, are also provided.

The present invention also relates to therapeutic
10 and diagnostic methods and compositions based on vertebrate Serrate proteins and nucleic acids. The invention provides for treatment of disorders of cell fate or differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds (termed herein "Therapeutics")
15 include: vertebrate Serrate proteins and analogs and derivatives (including fragments) thereof; antibodies thereto; nucleic acids encoding the vertebrate Serrate proteins, analogs, or derivatives; and vertebrate Serrate antisense nucleic acids. In a preferred embodiment, a
20 Therapeutic of the invention is administered to treat a cancerous condition, or to prevent progression from a pre-neoplastic or non-malignant state into a neoplastic or a malignant state. In other specific embodiments, a Therapeutic of the invention is administered to treat a
25 nervous system disorder or to promote tissue regeneration and repair.

In one embodiment, Therapeutics which antagonize, or inhibit, Notch and/or Serrate function (hereinafter "Antagonist Therapeutics") are administered for therapeutic
30 effect. In another embodiment, Therapeutics which promote Notch and/or Serrate function (hereinafter "Agonist Therapeutics") are administered for therapeutic effect.

Disorders of cell fate, in particular hyperproliferative (e.g., cancer) or hypoproliferative
35 disorders, involving aberrant or undesirable levels of expression or activity or localization of Notch and/or

Serrate protein can be diagnosed by detecting such levels, as described more fully infra.

In a preferred aspect, a Therapeutic of the invention is a protein consisting of at least a fragment
5 (termed herein "adhesive fragment") of a vertebrate Serrate which mediates binding to a Notch protein or a fragment thereof.

3.1. DEFINITIONS

10 As used herein, underscoring or italicizing the name of a gene shall indicate the gene, in contrast to its encoded protein product which is indicated by the name of the gene in the absence of any underscoring. For example, "Serrate" shall mean the Serrate gene, whereas "Serrate"
15 shall indicate the protein product of the Serrate gene.

4. DESCRIPTION OF THE FIGURES

Figure 1. Nucleotide sequence (SEQ ID NO:1) and protein sequence (SEQ ID NO:2) of Human Serrate-1 (also known
20 as Human Jagged-1 (HJ1)).

Figure 2. "Complete" nucleotide sequence (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4) of Human Serrate-2 (also known as Human Jagged-2 (HJ2)) generated on the computer by combining the sequence of clones pBS15 and
25 pBS3-2 isolated from human fetal brain cDNA libraries. There is a deletion of approximately 120 nucleotides in the region of this sequence which encodes the portion of Human Serrate-2 between the signal sequence and the beginning of the DSL domain.

30 Figure 3. Nucleotide sequence (SEQ ID NO:5) of chick Serrate (C-Serrate) cDNA.

Figure 4. Amino acid sequence (SEQ ID NO:6) of C-Serrate (lacking the amino-terminus of the signal sequence). The putative cleavage site following the signal
35 s qu nce (marking the pr dicted amino-terminus of the mature prot in) is mark d with an arrowhead; the DSL domain is indicated by asterisks; the EGF-lik rep ats (ELRs) ar

underlined with dashed lines; the cysteine rich region between the ELRs and the transmembrane domain is marked between arrows, and the single transmembrane domain (between amino acids 1042 and 1066) is shown in bold.

5 Figure 5. Alignment of the amino terminal sequences of *Drosophila melanogaster* Delta (SEQ ID NO:7) and Serrate (SEQ ID NO:8) with C-Serrate (SEQ ID NO:6). The region shown extends from the end of the signal sequence to the end of the DSL domain. The DSL domain is indicated.

10 Identical amino acids in all three proteins are boxed.

Figure 6. Diagram showing the domain structures of *Drosophila* Delta and *Drosophila* Serrate compared with C-Serrate. The second cysteine-rich region just downstream of the EGF repeats, present only in C-Serrate and *Drosophila*
15 Serrate, is not shown. Hydrophobic regions are shown in black; DSL domains are checkered and EGF-like repeats are hatched.

5. DETAILED DESCRIPTION OF THE INVENTION

20 The present invention relates to nucleotide sequences of vertebrate Serrate genes, and amino acid sequences of their encoded proteins. The invention further relates to fragments and other derivatives, and analogs, of vertebrate Serrate proteins. Nucleic acids encoding such
25 fragments or derivatives are also within the scope of the invention. The invention provides vertebrate Serrate genes and their encoded proteins of many different species. The Serrate genes of the invention include human Serrate and related genes (homologs) in vertebrate species. In specific
30 embodiments, the Serrate genes and proteins are from mammals. In a preferred embodiment of the invention, the Serrate protein is a human protein. In most preferred embodiments, the Serrate protein is Human Serrate-1 or Human Serrate-2. Production of the foregoing proteins and derivatives, e.g.,
35 by recombinant methods, is provided.

The invention relates to vertebrate Serrate derivatives and analogs of the invention which are

functionally active, i.e., they are capable of displaying one or more known functional activities associated with a full-length (wild-type) Serrate protein. Such functional activities include but are not limited to antigenicity
5 [ability to bind (or compete with Serrate for binding) to an anti-Serrate antibody], immunogenicity (ability to generate antibody which binds to Serrate), ability to bind (or compete with Serrate for binding) to Notch or other toporythmic proteins or fragments thereof ("adhesiveness"), ability to
10 bind (or compete with Serrate for binding) to a receptor for Serrate. "Toporythmic proteins" as used herein, refers to the protein products of *Notch*, *Delta*, *Serrate*, *Enhancer of split*, and *Deltex*, as well as other members of this interacting gene family which may be identified, e.g., by
15 virtue of the ability of their gene sequences to hybridize, or their homology to *Delta*, *Serrate*, or *Notch*, or the ability of their genes to display phenotypic interactions.

The invention further relates to fragments (and derivatives and analogs thereof) of a vertebrate Serrate
20 which comprise one or more domains of the Serrate protein, including but not limited to the intracellular domain, extracellular domain, transmembrane domain, membrane-associated region, or one or more EGF-like (homologous) repeats of a Serrate protein, or any combination of the
25 foregoing.

Antibodies to Serrate, its derivatives and analogs, are additionally provided.

As demonstrated *infra*, Serrate plays a critical role in development and other physiological processes, in
30 particular, as a ligand to Notch, which is involved in cell fate (differentiation) determination. In particular, Serrate is believed to play a major role in determining cell fates in the central nervous system. The nucleic acid and amino acid sequences and antibodies thereto of the invention can be used
35 for the detection and quantitation of Serrate mRNA and protein of human and other species, to study expression thereof, to produce Serrate and fragments and other

derivatives and analogs thereof, in the study and manipulation of differentiation and other physiological processes. The present invention also relates to therapeutic and diagnostic methods and compositions based on Serrate proteins and nucleic acids. The invention provides for treatment of disorders of cell fate or differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds (termed herein "Therapeutics") include: vertebrate Serrate proteins and analogs and derivatives (including fragments) thereof; antibodies thereto; nucleic acids encoding the vertebrate Serrate proteins, analogs, or derivatives; and vertebrate Serrate antisense nucleic acids. In a preferred embodiment, a Therapeutic of the invention is administered to treat a cancerous condition, or to prevent progression from a pre-neoplastic or non-malignant state into a neoplastic or a malignant state. In other specific embodiments, a Therapeutic of the invention is administered to treat a nervous system disorder or to promote tissue regeneration and repair.

In one embodiment, Therapeutics which antagonize, or inhibit, Notch and/or Serrate function (hereinafter "Antagonist Therapeutics") are administered for therapeutic effect. In another embodiment, Therapeutics which promote Notch and/or Serrate function (hereinafter "Agonist Therapeutics") are administered for therapeutic effect.

Disorders of cell fate, in particular hyperproliferative (e.g., cancer) or hypoproliferative disorders, involving aberrant or undesirable levels of expression or activity or localization of Notch and/or Serrate protein can be diagnosed by detecting such levels, as described more fully *infra*.

In a preferred aspect, a Therapeutic of the invention is a protein consisting of at least a fragment (term d herein "adhesive fragment") of a vertebrate Serrate which mediates binding to a Notch protein or a fragment thereof.

The invention is illustrated by way of examples *infra* which disclose, *inter alia*, the cloning of a mouse Serrate homolog (Section 6), the cloning of a *Xenopus* (frog) Serrate homolog (Section 7), the cloning of a chick Serrate homolog (Section 8), and the cloning of the human Serrate homologs *Human Serrate-1* (HJ1) and *Human Serrate-2* (HJ2) (Section 9).

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the sub-sections which follow.

5.1. ISOLATION OF THE SERRATE GENES

The invention relates to the nucleotide sequences of vertebrate Serrate nucleic acids. In specific embodiments, vertebrate Serrate nucleic acids comprise the cDNA sequences shown in Figure 1 (SEQ ID NO:1), Figure 2 (SEQ ID NO:3), Figure 3 (SEQ ID NO:6) or the coding regions thereof, or nucleic acids encoding a vertebrate Serrate protein (e.g., having the sequence of SEQ ID NO:2, 4, or 6).

The invention provides nucleic acids consisting of at least 8 nucleotides (i.e., a hybridizable portion) of a vertebrate Serrate sequence; in other embodiments, the nucleic acids consist of at least 10 (continuous) nucleotides, 25 nucleotides, 50 nucleotides, 100 nucleotides, 150 nucleotides, or 200 nucleotides of a vertebrate Serrate sequence, or a full-length vertebrate Serrate coding sequence. The invention also relates to nucleic acids hybridizable to or complementary to the foregoing sequences. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of a Serrate gene.

In a specific embodiment, a nucleic acid which is hybridizable to a vertebrate Serrate nucleic acid (e.g., having sequence SEQ ID NO:1), or to a nucleic acid encoding a vertebrate Serrate derivative, under conditions of low stringency is provided. By way of example and not

limitation, procedures using such conditions of low stringency are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 h at 40°C in a solution containing 5 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) 10 dextran sulfate, and 5-20 X 10⁶ cpm ³²P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 h at 40°C, and then washed for 1.5 h at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution 15 and incubated an additional 1.5 h at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film. Other conditions of low stringency which may be used are well known in the art (e.g., as employed for cross- 20 species hybridizations).

In another specific embodiment, a nucleic acid which is hybridizable to a vertebrate Serrate nucleic acid under conditions of high stringency is provided. By way of example and not limitation, procedures using such conditions 25 of high stringency are as follows: Prehybridization of filters containing DNA is carried out for 8 h to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 h 30 at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10⁶ cpm of ³²P-labeled probe. Washing of filters is done at 37°C for 1 h in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1X SSC at 50°C 35 for 45 min before autoradiography. Other conditions of high stringency which may be used are well known in the art.

Nucleic acids encoding fragments and derivatives of vertebrate Serrate proteins (see Section 5.6), and vertebrate Serrate antisense nucleic acids (see Section 5.11) are additionally provided. As is readily apparent, as used herein, a "nucleic acid encoding a fragment or portion of a Serrate protein" shall be construed as referring to a nucleic acid encoding only the recited fragment or portion of the Serrate protein and not the other contiguous portions of the Serrate protein as a continuous sequence.

10 Fragments of vertebrate Serrate nucleic acids comprising regions of homology to other toporythmic proteins are also provided. The DSL regions (regions of homology with *Drosophila* Delta and Serrate) of Serrate proteins of other species are also provided. Nucleic acids encoding conserved
15 regions between Delta and Serrate, such as those represented by Serrate amino acids 63-73, 124-134, 149-158, 195-206, 214-219, and 250-259 of SEQ ID NO:8, or by the DSL domains are also provided.

Specific embodiments for the cloning of a
20 vertebrate Serrate gene, presented as a particular example but not by way of limitation, follows:

For expression cloning (a technique commonly known in the art), an expression library is constructed by methods known in the art. For example, mRNA (e.g., human) is
25 isolated, cDNA is made and ligated into an expression vector (e.g., a bacteriophage derivative) such that it is capable of being expressed by the host cell into which it is then introduced. Various screening assays can then be used to select for the expressed Serrate product. In one embodiment,
30 anti-Serrate antibodies can be used for selection.

In another preferred aspect, PCR is used to amplify the desired sequence in a genomic or cDNA library, prior to selection. Oligonucleotide primers representing known Serrate sequences can be used as primers in PCR. In a
35 preferred aspect, the oligonucleotide primers encode at least part of the Serrate conserved segments of strong homology between Serrate and Delta. The synthetic oligonucleotides

may be utilized as primers to amplify by PCR sequences from a source (RNA or DNA), preferably a cDNA library, of potential interest. PCR can be carried out, e.g., by use of a Perkin-Elmer Cetus thermal cyclor and Taq polymerase (Gene Amp[™]).

5 The DNA being amplified can include mRNA or cDNA or genomic DNA from any eukaryotic species. One can choose to synthesize several different degenerate primers, for use in the PCR reactions. It is also possible to vary the stringency of hybridization conditions used in priming the

10 PCR reactions, to allow for greater or lesser degrees of nucleotide sequence similarity between the known Serrate nucleotide sequence and the nucleic acid homolog being isolated. For cross species hybridization, low stringency conditions are preferred. For same species hybridization,

15 moderately stringent conditions are preferred. After successful amplification of a segment of a Serrate homolog, that segment may be cloned and sequenced, and utilized as a probe to isolate a complete cDNA or genomic clone. This, in turn, will permit the determination of the gene's complete

20 nucleotide sequence, the analysis of its expression, and the production of its protein product for functional analysis, as described *infra*. In this fashion, additional genes encoding Serrate proteins may be identified. Such a procedure is presented by way of example in various examples sections

25 *infra*.

The above-methods are not meant to limit the following general description of methods by which clones of vertebrate Serrate may be obtained.

Any vertebrate cell potentially can serve as the

30 nucleic acid source for the molecular cloning of the Serrate gene. The nucleic acid sequences encoding Serrate can be isolated from human, porcine, bovine, feline, avian, equine, canine, as well as additional primate sources, etc. For example, we have amplified fragments of the appropriate size

35 in mouse, *Xenopus*, and human, by PCR using cDNA libraries with *Drosophila* Serrate primers. The DNA may be obtained by standard procedures known in the art from cloned DNA (e.g., a

DNA "library"), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA, or fragments thereof, purified from the desired cell. (See, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.) Clones derived from genomic DNA may contain regulatory and intron DNA regions in addition to coding regions; clones derived from cDNA will contain only exon sequences. Whatever the source, the gene should be molecularly cloned into a suitable vector for propagation of the gene.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will encode the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNase in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis and column chromatography.

Once the DNA fragments are generated, identification of the specific DNA fragment containing the desired gene may be accomplished in a number of ways. For example, if a *Serrate* (of any species) gene or its specific RNA, or a fragment thereof, e.g., an extracellular domain (see Section 5.6), is available and can be purified and labeled, the generated DNA fragments may be screened by nucleic acid hybridization to the labeled probe (Benton, W. and Davis, R., 1977, Science 196:180; Grunstein, M. And Hogness, D., 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further

selection can be carried out on the basis of the properties of the gene. Alternatively, the presence of the gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected which produce a protein that, e.g., has similar or identical electrophoretic migration, isoelectric focusing behavior, proteolytic digestion maps, receptor binding activity, *in vitro* aggregation activity ("adhesiveness") or antigenic properties as known for Serrate. If an antibody to Serrate is available, the Serrate protein may be identified by binding of labeled antibody to the putatively Serrate synthesizing clones, in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

The Serrate gene can also be identified by mRNA selection by nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Such DNA fragments may represent available, purified Serrate DNA of another species (e.g., human, chick). Immunoprecipitation analysis or functional assays (e.g., aggregation ability *in vitro*; binding to receptor; see *infra*) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences. In addition, specific mRNAs may be selected by adsorption of polysomes isolated from cells to immobilized antibodies specifically directed against Serrate protein. A radiolabeled Serrate cDNA can be synthesized using the selected mRNA (from the adsorbed polysomes) as a template. The radiolabeled mRNA or cDNA may then be used as a probe to identify the Serrate DNA fragments from among other genomic DNA fragments.

Alternatives to isolating the Serrate genomic DNA include, but are not limited to, chemically synthesizing the gene sequence itself from a known sequence or making cDNA to the mRNA which encodes the Serrate protein. For example, RNA

for cDNA cloning of the Serrate gene can be isolated from cells which express Serrate. Other methods are possible and within the scope of the invention.

The identified and isolated gene can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives. The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and Serrate gene may be modified by homopolymeric tailing. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc., so that many copies of the gene sequence are generated.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionization, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate the isolated Serrate gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from

the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The Serrate sequences provided by the instant invention include those nucleotide sequences encoding substantially the same amino acid sequences as found in native Serrate proteins, and those encoded amino acid sequences with functionally equivalent amino acids, all as described in Section 5.6 *infra* for Serrate derivatives.

10

5.2. EXPRESSION OF THE SERRATE GENES

The nucleotide sequence coding for a vertebrate Serrate protein or a functionally active fragment or other derivative thereof (see Section 5.6), can be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native vertebrate Serrate gene and/or its flanking regions. A variety of host-vector systems may be utilized to express the protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA. The expression elements of vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used. In a specific embodiment, the adhesive portion of the Serrate gene is expressed. In other specific embodiments, a Human Serrate gene or a sequence encoding a functionally active portion of a human Serrate gene, such as Human Serrate-1 (HJ2) or Human Serrate-2 (HJ2), is expressed. In yet another embodiment, a fragment of Serrate comprising the extracellular domain, or other derivative, or analog of Serrate is expressed.

Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinants (genetic recombination). Expression of nucleic acid sequence encoding a Serrate protein or peptide fragment may be regulated by a second nucleic acid sequence so that the Serrate protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of a Serrate protein may be controlled by any promoter/enhancer element known in the art. Promoters which may be used to control toporythmic gene expression include, but are not limited to, the SV40 early promoter region (Beruoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the *tac* promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25); see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter of the photosynthetic enzyme ribulose biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following animal transcriptional control

regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409; MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538; Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58; alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171), beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing Serrate gene inserts can be identified by three general approaches: (a) nucleic acid hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by nucleic acid hybridization using probes comprising sequences that are homologous to an inserted toporythmic gene. In the second

approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the Serrate gene is inserted within the marker gene sequence of the vector, recombinants containing the Serrate insert can be identified by the absence of the marker gene function. In the third approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant. Such assays can be based, for example, on the physical or functional properties of the Serrate gene product *in vitro* assay systems, e.g., aggregation (binding) with Notch, binding to a receptor, binding with antibody.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered Serrate protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g.,

glycosylation, cleavage [e.g., of signal sequence]) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in yeast will produce a glycosylated product. Expression in mammalian cells can be used to ensure "native" glycosylation of a heterologous mammalian toporythmic protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

In other specific embodiments, the Serrate protein, fragment, analog, or derivative may be expressed as a fusion, or chimeric protein product (comprising the protein, fragment, analog, or derivative joined via a peptide bond to a heterologous protein sequence (of a different protein)). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, e.g., by use of a peptide synthesizer.

Both cDNA and genomic sequences can be cloned and expressed.

5.3. IDENTIFICATION AND PURIFICATION OF THE SERRATE GENE PRODUCTS

In particular aspects, the invention provides amino acid sequences of a vertebrate Serrate, preferably a human Serrate homolog, and fragments and derivatives thereof which comprise an antigenic determinant (i.e., can be recognized by an antibody) or which are otherwise functionally active, as well as nucleic acid sequences encoding the foregoing. "Functionally active" material as used herein refers to that material displaying one or more known functional activities associated with a full-length (wild-type) Serrate protein,

e.g., binding to Notch or a portion thereof, binding to any other Serrate ligand, antigenicity (binding to an anti-Serrate antibody), etc.

In specific embodiments, the invention provides 5 fragments of a vertebrate Serrate protein consisting of at least 6 amino acids, 10 amino acids, 25 amino acids, 50 amino acids, or of at least 75 amino acids. In other embodiments, the proteins comprise or consist essentially of an
10 extracellular domain, DSL domain, epidermal growth factor-like repeat (ELR) domain, one or any combination of ELRs, cysteine-rich region, transmembrane domain, or intracellular (cytoplasmic) domain, or a portion which binds to Notch, or any combination of the foregoing, of a Serrate protein. Fragments, or proteins comprising fragments, lacking some or
15 all of the foregoing regions of a vertebrate Serrate protein are also provided. Nucleic acids encoding the foregoing are provided.

Once a recombinant which expresses the vertebrate Serrate gene sequence is identified, the gene product can be
20 analyzed. This is achieved by assays based on the physical or functional properties of the product, including radioactive labelling of the product followed by analysis by gel electrophoresis, immunoassay, etc.

Once the Serrate protein is identified, it may be
25 isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. The functional properties may be
30 evaluated using any suitable assay (see Section 5.7).

Alternatively, once a Serrate protein produced by a recombinant is identified, the amino acid sequence of the protein can be deduced from the nucleotide sequence of the chimeric gene contained in the recombinant. As a result, the
35 protein can be synthesized by standard chemical methods known in the art (e.g., see Hunkapiller, M., et al., 1984, Nature 310:105-111).

In a specific embodiment of the present invention, such Serrate proteins, whether produced by recombinant DNA techniques or by chemical synthetic methods, include but are not limited to those containing, as a primary amino acid sequence, all or part of the amino acid sequence substantially as depicted in Figures 1, 2, or 3 (SEQ ID NO:2, 4, or 6, respectively), as well as fragments and other derivatives, and analogs thereof.

10 5.4. STRUCTURE OF THE SERRATE GENES AND PROTEINS

The structure of the Serrate genes and proteins can be analyzed by various methods known in the art.

 5.4.1. GENETIC ANALYSIS

15 The cloned DNA or cDNA corresponding to the vertebrate Serrate gene can be analyzed by methods including but not limited to Southern hybridization (Southern, E.M., 1975, J. Mol. Biol. 98:503-517), Northern hybridization (see e.g., Freeman et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:4094-4098), restriction endonuclease mapping (Maniatis, T., 1982, Molecular Cloning, A Laboratory, Cold Spring Harbor, New York), and DNA sequence analysis. Polymerase chain reaction (PCR; U.S. Patent Nos. 4,683,202, 4,683,195 and 4,889,818; Gyllenstein et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7652-7656; Ochman et al., 1988, Genetics 120:621-623; Loh et al., 1989, Science 243:217-220) followed by Southern hybridization with a Serrate-specific probe can allow the detection of the Serrate gene in DNA from various cell types. Methods of amplification other than PCR are
20 commonly known and can also be employed. In one embodiment, Southern hybridization can be used to determine the genetic linkage of Serrate. Northern hybridization analysis can be used to determine the expression of the Serrate gene. Various cell types, at various states of development or
25 activity can be tested for Serrate expression. Examples of such techniques and their results are described in Section 6, *infra*. The stringency of the hybridization conditions for

both Southern and Northern hybridization can be manipulated to insure detection of nucleic acids with the desired degree of relatedness to the specific Serrate probe used.

Restriction endonuclease mapping can be used to roughly determine the genetic structure of the Serrate gene. In a particular embodiment, cleavage with restriction enzymes can be used to derive the restriction map shown in Figure 2, *infra*. Restriction maps derived by restriction endonuclease cleavage can be confirmed by DNA sequence analysis.

10 DNA sequence analysis can be performed by any techniques known in the art, including but not limited to the method of Maxam and Gilbert (1980, Meth. Enzymol. 65:499-560), the Sanger dideoxy method (Sanger, F., et al., 1977, Proc. Natl. Acad. Sci. U.S.A. 74:5463), the use of T7 DNA
15 polymerase (Tabor and Richardson, U.S. Patent No. 4,795,699), or use of an automated DNA sequenator (e.g., Applied Biosystems, Foster City, CA). The cDNA sequence of a representative Serrate gene comprises the sequence substantially as depicted in Figures 1 and 2, and is
20 described in Section 9, *infra*.

5.4.2. PROTEIN ANALYSIS

The amino acid sequence of the Serrate proteins can be derived by deduction from the DNA sequence, or
25 alternatively, by direct sequencing of the protein, e.g., with an automated amino acid sequencer. The amino acid sequence of a representative Serrate protein comprises the sequence substantially as depicted in Figure 1, and detailed in Section 9, *infra*, with the representative mature protein
30 that shown by amino acid numbers 30-1219.

The Serrate protein sequence can be further characterized by a hydrophilicity analysis (Hopp, T. and Woods, K., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824). A hydrophilicity profile can be used to identify the
35 hydrophobic and hydrophilic regions of the Serrate protein and the corresponding regions of the gene sequence which encode such regions.

Secondary, structural analysis (Chou, P. and Fasman, G., 1974, Biochemistry 13:222) can also be done, to identify regions of Serrate that assume specific secondary structures.

5 Manipulation, translation, and secondary structure prediction, as well as open reading frame prediction and plotting, can also be accomplished using computer software programs available in the art.

Other methods of structural analysis can also be
10 employed. These include but are not limited to X-ray crystallography (Engstrom, A., 1974, Biochem. Exp. Biol. 11:7-13) and computer modeling (Fletterick, R. and Zoller, M. (eds.), 1986, Computer Graphics and Molecular Modeling, in Current Communications in Molecular Biology, Cold Spring
15 Harbor Laboratory, Cold Spring Harbor, New York).

5.5. GENERATION OF ANTIBODIES TO SERRATE PROTEINS AND DERIVATIVES THEREOF

According to the invention, a vertebrate Serrate
20 protein, its fragments or other derivatives, or analogs thereof, may be used as an immunogen to generate antibodies which recognize such an immunogen. Such antibodies include but are not limited to polyclonal, monoclonal, chimeric, single chain, Fab fragments, and an Fab expression library.
25 In a specific embodiment, antibodies to human Serrate are produced. In another embodiment, antibodies to the extracellular domain of Serrate are produced. In another embodiment, antibodies to the intracellular domain of Serrate are produced.

30 Various procedures known in the art may be used for the production of polyclonal antibodies to a Serrate protein or derivative or analog. In a particular embodiment, rabbit polyclonal antibodies to an epitope of the Serrate protein encoded by a sequence depicted in Figure 1, or a subsequence
35 thereof, can be obtained. For the production of antibody, various host animals can be immunized by injection with the native Serrate protein, or a synthetic version, or derivative

(e.g., fragment) thereof, including but not limited to rabbits, mice, rats, etc. Various adjuvants may be used to increase the immunological response, depending on the host species, and including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum.

For preparation of monoclonal antibodies directed toward a vertebrate Serrate protein sequence or analog thereof, any technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497), as well as the trioma technique, the human B-cell hybridoma technique (Kozbor et al., 1983, Immunology Today 4:72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention, monoclonal antibodies can be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:2026-2030) or by transforming human B cells with EBV virus in vitro (Cole et al., 1985, in Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci. U.S.A. 81:6851-6855; Neuberger et al., 1984, Nature 312:604-608; Takeda et al., 1985, Nature 314:452-454) by splicing the genes from a mouse antibody molecule specific for Serrate together with genes from a human antibody molecule of appropriate biological activity

can be used; such antibodies are within the scope of this invention.

According to the invention, techniques described for the production of single chain antibodies (U.S. Patent 5 4,946,778) can be adapted to produce Serrate-specific single chain antibodies. An additional embodiment of the invention utilizes the techniques described for the construction of Fab expression libraries (Huse et al., 1989, Science 246:1275-1281) to allow rapid and easy identification of monoclonal 10 Fab fragments with the desired specificity for Serrate proteins, derivatives, or analogs.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For example, such fragments include but are not limited to: the 15 F(ab'), fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab'), fragment, and the Fab fragments which can be generated by treating the antibody molecule with papain and a reducing 20 agent.

In the production of antibodies, screening for the desired antibody can be accomplished by techniques known in the art, e.g. ELISA (enzyme-linked immunosorbent assay). For example, to select antibodies which recognize a specific 25 domain of a Serrate protein, one may assay generated hybridomas for a product which binds to a Serrate fragment containing such domain. For selection of an antibody specific to vertebrate (e.g., human) Serrate, one can select on the basis of positive binding to vertebrate Serrate and a 30 lack of binding to *Drosophila* Serrate. In another embodiment, one can select for binding to human Serrate and not to Serrate of other species.

The foregoing antibodies can be used in methods known in the art relating to the localization and activity of 35 the protein sequences of the invention (e.g., see Section 5.7, *infra*), e.g., for imaging these proteins, measuring

levels thereof in appropriate physiological samples, in diagnostic methods, etc.

Antibodies specific to a domain of a Serrate protein are also provided. In a specific embodiment, 5 antibodies which bind to a Notch-binding fragment of Serrate are provided.

In another embodiment of the invention (see *infra*), anti-Serrate antibodies and fragments thereof containing the binding domain are Therapeutics.

10

5.6. SERRATE PROTEINS, DERIVATIVES AND ANALOGS

The invention further relates to vertebrate Serrate proteins, and derivatives (including but not limited to fragments) and analogs of Serrate proteins. Nucleic acids 15 encoding vertebrate Serrate protein derivatives and protein analogs are also provided. In one embodiment, the Serrate proteins are encoded by the vertebrate Serrate nucleic acids described in Section 5.1 *supra*. In particular aspects, the proteins, derivatives, or analogs are of frog, mouse, rat, 20 pig, cow, dog, monkey, or human Serrate proteins.

The production and use of derivatives and analogs related to vertebrate Serrate are within the scope of the present invention. In a specific embodiment, the derivative or analog is functionally active, i.e., capable of exhibiting 25 one or more functional activities associated with a full-length, wild-type Serrate protein. As one example, such derivatives or analogs which have the desired immunogenicity or antigenicity can be used, for example, in immunoassays, for immunization, for inhibition of Serrate activity, etc. 30 Such molecules which retain, or alternatively inhibit, a desired Serrate property, e.g., binding to Notch or other toporythmic proteins, binding to a cell-surface receptor, can be used as inducers, or inhibitors, respectively, of such property and its physiological correlates. A specific 35 embodiment relates to a Serrate fragment that can be bound by an anti-S rrat antibody but cannot bind to a Notch protein or oth r toporythmic protein. Derivatives or analogs of

Serrate can be tested for the desired activity by procedures known in the art, including but not limited to the assays described in Section 5.7.

In particular, Serrate derivatives can be made by
5 altering Serrate sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a Serrate gene may be used in the practice of the
10 present invention. These include but are not limited to nucleotide sequences comprising all or portions of Serrate genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change.
15 Likewise, the Serrate derivatives of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a Serrate protein including altered sequences in which functionally equivalent amino acid residues are substituted
20 for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino
25 acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids
30 include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.
35 In a specific embodiment of the invention, proteins consisting of or comprising a fragment of a vertebrate Serrate protein consisting of at least 10 (continuous) amino

acids of the Serrate protein is provided. In other embodiments, the fragment consists of at least 20 or 50 amino acids of the Serrate protein. In specific embodiments, such fragments are not larger than 35, 100 or 200 amino acids.

- 5 Derivatives or analogs of vertebrate Serrate include but are not limited to those peptides which are substantially homologous to a vertebrate Serrate or a fragment thereof (e.g., at least 30% identity over an amino acid sequence of identical size) or whose encoding nucleic acid is capable of
10 hybridizing to a coding vertebrate Serrate sequence.

- The Serrate derivatives and analogs of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at the gene or protein level. For example, the cloned
15 Serrate gene sequence can be modified by any of numerous strategies known in the art (Maniatis, T., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). The sequence can be cleaved at appropriate sites with restriction
20 endonuclease(s), followed by further enzymatic modification if desired, isolated, and ligated *in vitro*. In the production of the gene encoding a derivative or analog of Serrate, care should be taken to ensure that the modified gene remains within the same translational reading frame as
25 Serrate, uninterrupted by translational stop signals, in the gene region where the desired Serrate activity is encoded.

- Additionally, the Serrate-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination
30 sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including but not limited to, *in vitro* site-
35 directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB linkers (Pharmacia), etc.

Manipulations of the Serrate sequence may also be made at the protein level. Included within the scope of the invention are Serrate protein fragments or other derivatives or analogs which are differentially modified during or after translation, e.g., by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc. Any of numerous chemical modifications may be carried out by known techniques, including but not limited to specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH_4 ; acetylation, formylation, oxidation, reduction; metabolic synthesis in the presence of tunicamycin; etc.

In addition, analogs and derivatives of Serrate can be chemically synthesized. For example, a peptide corresponding to a portion of a Serrate protein which comprises the desired domain (see Section 5.6.1), or which mediates the desired aggregation activity *in vitro*, or binding to a receptor, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the Serrate sequence. Non-classical amino acids include but are not limited to the D-isomers of the common amino acids, α -amino isobutyric acid, 4-aminobutyric acid, hydroxyproline, sarcosine, citrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β -alanine, designer amino acids such as β -methyl amino acids, $\text{C}\alpha$ -methyl amino acids, and $\text{N}\alpha$ -methyl amino acids.

In a specific embodiment, the Serrate derivative is a chimeric, or fusion, protein comprising a vertebrate Serrate protein or fragment thereof (preferably consisting of at least a domain or motif of the Serrate protein, or at least 10 amino acids of the Serrate protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment,

such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a Serrate-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by
5 ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein
10 synthetic techniques, e.g., by use of a peptide synthesizer. In a specific embodiment, a chimeric nucleic acid encoding a mature vertebrate Serrate protein with a heterologous signal sequence is expressed such that the chimeric protein is expressed and processed by the cell to the mature Serrate
15 protein. As another example, and not by way of limitation, a recombinant molecule can be constructed according to the invention, comprising coding portions of both Serrate and another toporythmic gene, e.g., Delta. The encoded protein of such a recombinant molecule could exhibit properties
20 associated with both Serrate and Delta and portray a novel profile of biological activities, including agonists as well as antagonists. The primary sequence of Serrate and Delta may also be used to predict tertiary structure of the molecules using computer simulation (Hopp and Woods, 1981,
25 Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828); Serrate/Delta chimeric recombinant genes could be designed in light of correlations between tertiary structure and biological function. Likewise, chimeric genes comprising portions of a vertebrate Serrate fused to any heterologous protein-encoding
30 sequences may be constructed. A specific embodiment relates to a chimeric protein comprising a fragment of a vertebrate Serrate of at least ten amino acids.

In another specific embodiment, the Serrate derivative is a fragment of Serrate comprising a region of
35 homology with another toporythmic protein. As used herein, a region of a first protein shall be considered "homologous" to a second prot in when the amino acid sequence of the region

is at least 30% identical or at least 75% either identical or involving conservative changes, when compared to any sequence in the second protein of an equal number of amino acids as the number contained in the region. For example, such a Serrate fragment can comprise one or more regions homologous to Delta, or DSL domains or portions thereof.

Other specific embodiments of derivatives and analogs are described in the subsections below and examples sections *infra*.

10

5.6.1. DERIVATIVES OF SERRATE CONTAINING ONE OR MORE DOMAINS OF THE PROTEIN

In a specific embodiment, the invention relates to vertebrate Serrate derivatives and analogs, in particular vertebrate Serrate fragments and derivatives of such fragments, that comprise, or alternatively consist of, one or more domains of the Serrate protein, including but not limited to the extracellular domain, DSL domain, ELR domain, cysteine rich domain, transmembrane domain, intracellular domain, membrane-associated region, and one or more of the EGF-like repeats (ELR) of the Serrate protein, or any combination of the foregoing. In particular examples relating to the human and chick Serrate proteins, such domains are identified in Examples Section 9 and 8, respectively.

25

In a specific embodiment, the molecules comprising specific fragments of vertebrate Serrate are those comprising fragments in the respective Serrate protein most homologous to specific fragments of the *Drosophila* Serrate and/or Delta proteins. In particular embodiments, such a molecule comprises or consists of the amino acid sequences homologous to SEQ ID NO:10, 12, or 18. Alternatively, a fragment comprising a domain of a Serrate homolog can be identified by protein analysis methods as described in Section 5.3.2.

35

5.6.2. DERIVATIVES OF SERRATE THAT MEDIATE BINDING TO TOPORYTHMIC PROTEIN DOMAINS

The invention also provides for vertebrate Serrate fragments, and analogs or derivatives of such fragments, which mediate binding to toporythmic proteins (and thus are termed herein "adhesive"), and nucleic acid sequences encoding the foregoing.

In a specific embodiment, the adhesive fragment of Serrate is that comprising the portion of Serrate most homologous to about amino acid numbers 85-283 or 79-282 of the *Drosophila* Serrate sequence (see PCT Publication WO 93/12141 dated June 24, 1993).

In a particular embodiment, the adhesive fragment of a Serrate protein comprises the DSL domain, or a portion thereof. Subfragments within the DSL domain that mediate binding to Notch can be identified by analysis of constructs expressing deletion mutants.

The ability to bind to a toporythmic protein (preferably Notch) can be demonstrated by *in vitro* aggregation assays with cells expressing such a toporythmic protein as well as cells expressing Serrate or a Serrate derivative (See Section 5.7). That is, the ability of a Serrate fragment to bind to a Notch protein can be demonstrated by detecting the ability of the Serrate fragment, when expressed on the surface of a first cell, to bind to a Notch protein expressed on the surface of a second cell.

The nucleic acid sequences encoding toporythmic proteins or adhesive domains thereof, for use in such assays, can be isolated from human, porcine, bovine, feline, avian, equine, canine, or insect, as well as primate sources and any other species in which homologs of known toporythmic genes can be identified.

35

5.7. ASSAYS OF SERRATE PROTEINS, DERIVATIVES AND ANALOGS

The functional activity of vertebrate Serrate proteins, derivatives and analogs can be assayed by various methods.

For example, in one embodiment, where one is assaying for the ability to bind or compete with wild-type Serrate for binding to anti-Serrate antibody, various immunoassays known in the art can be used, including but not limited to competitive and non-competitive assay systems using techniques such as radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitation reactions, immunodiffusion assays, *in situ* immunoassays (using colloidal gold, enzyme or radioisotope labels, for example), western blots, precipitation reactions, agglutination assays (e.g., gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is detected by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by detecting binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

In another embodiment, where one is assaying for the ability to mediate binding to a toporythmic protein, e.g., Notch, one can carry out an *in vitro* aggregation assay such as described in PCT Publication WO 93/12141 dated June 24, 1993 (see also Fehon et al., 1990, Cell 61:523-534; Rebay et al., 1991, Cell 67:687-699).

In another embodiment, where a receptor for Serrate is identified, receptor binding can be assayed, e.g., by means well-known in the art. In another embodiment, physiological correlates of Serrate binding to cells

expressing a Serrate receptor (signal transduction) can be assayed.

In another embodiment, in insect or other model systems, genetic studies can be done to study the phenotypic effect of a Serrate mutant that is a derivative or analog of wild-type vertebrate Serrate.

Other methods will be known to the skilled artisan and are within the scope of the invention.

10

5.8. THERAPEUTIC USES

The invention provides for treatment of disorders of cell fate or differentiation by administration of a therapeutic compound of the invention. Such therapeutic compounds (termed herein "Therapeutics") include: vertebrate Serrate proteins and analogs and derivatives (including fragments) thereof (e.g., as described hereinabove); antibodies thereto (as described hereinabove); nucleic acids encoding the vertebrate Serrate proteins, analogs, or derivatives (e.g., as described hereinabove); and Serrate antisense nucleic acids. As stated *supra*, the Antagonist Therapeutics of the invention are those Therapeutics which antagonize, or inhibit, a vertebrate Serrate function and/or Notch function (since Serrate is a Notch ligand). Such Antagonist Therapeutics are most preferably identified by use of known convenient *in vitro* assays, e.g., based on their ability to inhibit binding of Serrate to another protein (e.g., a Notch protein), or inhibit any known Notch or Serrate function as preferably assayed *in vitro* or in cell culture, although genetic assays (e.g., in *Drosophila*) may also be employed. In a preferred embodiment, the Antagonist Therapeutic is a protein or derivative thereof comprising a functionally active fragment such as a fragment of Serrate which mediates binding to Notch, or an antibody thereto. In other specific embodiments, such an Antagonist Therapeutic is a nucleic acid capable of expressing a molecule comprising a fragment of Serrate which binds to Notch, or a Serrate antisense nucleic acid (see Section 5.11 herein). It should

be noted that preferably, suitable *in vitro* or *in vivo* assays, as described *infra*, should be utilized to determine the effect of a specific Therapeutic and whether its administration is indicated for treatment of the affected
5 tissue, since the developmental history of the tissue may determine whether an Antagonist or Agonist Therapeutic is desired.

In addition, the mode of administration, e.g., whether administered in soluble form or administered via its
10 encoding nucleic acid for intracellular recombinant expression, of the Serrate protein or derivative can affect whether it acts as an agonist or antagonist.

In another embodiment of the invention, a nucleic acid containing a portion of a vertebrate Serrate gene is
15 used, as an Antagonist Therapeutic, to promote Serrate inactivation by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

The Agonist Therapeutics of the invention, as
20 described *supra*, promote Serrate function. Such Agonist Therapeutics include but are not limited to proteins and derivatives comprising the portions of Notch that mediate binding to Serrate, and nucleic acids encoding the foregoing (which can be administered to express their encoded products
25 *in vivo*).

Further descriptions and sources of Therapeutics of the inventions are found in Sections 5.1 through 5.7 herein.

Molecules which retain, or alternatively inhibit, a desired Serrate property, e.g., binding to Notch, binding to
30 an intracellular ligand, can be used therapeutically as inducers, or inhibitors, respectively, of such property and its physiological correlates. In a specific embodiment, a peptide (e.g., in the range of 10-50 or 15-25 amino acids; and particularly of about 10, 15, 20 or 25 amino acids)
35 containing the sequence of a portion of a vertebrate Serrate which binds to Notch is used to antagonize Notch function. In a specific embodiment, such an Antagonist Therapeutic is

used to treat or prevent human or other malignancies associated with increased Notch expression (e.g., cervical cancer, colon cancer, breast cancer, squamous adenocarcinomas (see *infra*)). Derivatives or analogs of Serrate can be
5 tested for the desired activity by procedures known in the art, including but not limited to the assays described in the examples *infra*. For example, molecules comprising vertebrate Serrate fragments which bind to Notch EGF-repeats (ELR) 11 and 12 and which are smaller than a DSL domain, can be
10 obtained and selected by expressing deletion mutants and assaying for binding of the expressed product to Notch by any of the several methods (e.g., *in vitro* cell aggregation assays, interaction trap system), some of which are described in the Examples Sections *infra*. In one specific embodiment,
15 peptide libraries can be screened to select a peptide with the desired activity; such screening can be carried out by assaying, e.g., for binding to Notch or a molecule containing the Notch ELR 11 and 12 repeats.

The Agonist and Antagonist Therapeutics of the
20 invention have therapeutic utility for disorders of cell fate. The Agonist Therapeutics are administered therapeutically (including prophylactically): (1) in diseases or disorders involving an absence or decreased (relative to normal, or desired) levels of Notch or Serrate function, for
25 example, in patients where Notch or Serrate protein is lacking, genetically defective, biologically inactive or underactive, or underexpressed; and (2) in diseases or disorders wherein *in vitro* (or *in vivo*) assays (see *infra*) indicate the utility of Serrate agonist administration. The
30 absence or decreased levels in Notch or Serrate function can be readily detected, e.g., by obtaining a patient tissue sample (e.g., from biopsy tissue) and assaying it *in vitro* for protein levels, structure and/or activity of the expressed Notch or Serrate protein. Many methods standard in
35 the art can be thus employed, including but not limited to immunoassays to detect and/or visualize Notch or Serrate protein (e.g., Western blot, immunoprecipitation followed by

sodium dodecyl sulfate polyacrylamide gel electrophoresis, immunocytochemistry, etc.) and/or hybridization assays to detect Notch or Serrate expression by detecting and/or visualizing respectively Notch or Serrate mRNA (e.g.,

5 Northern assays, dot blots, *in situ* hybridization, etc.)

In vitro assays which can be used to determine whether administration of a specific Agonist Therapeutic or Antagonist Therapeutic is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in

10 culture, and exposed to or otherwise administered a Therapeutic, and the effect of such Therapeutic upon the tissue sample is observed. In one embodiment, where the patient has a malignancy, a sample of cells from such malignancy is plated out or grown in culture, and the cells

15 are then exposed to a Therapeutic. A Therapeutic which inhibits survival or growth of the malignant cells (e.g., by promoting terminal differentiation) is selected for therapeutic use *in vivo*. Many assays standard in the art can be used to assess such survival and/or growth; for example,

20 cell proliferation can be assayed by measuring ³H-thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto-oncogenes (e.g., *fos*, *myc*) or cell cycle markers; cell viability can be assessed by trypan blue staining,

25 differentiation can be assessed visually based on changes in morphology, etc. In a specific aspect, the malignant cell cultures are separately exposed to (1) an Agonist Therapeutic, and (2) an Antagonist Therapeutic; the result of the assay can indicate which type of Therapeutic has

30 therapeutic efficacy.

In another embodiment, a Therapeutic is indicated for use which exhibits the desired effect, inhibition or promotion of cell growth, upon a patient cell sample from tissue having or suspected of having a hyper- or

35 hypoproliferative disorder, respectively. Such hyper- or hypoproliferative disorders include but are not limited to those described in Sections 5.8.1 through 5.8.3 *infra*.

In another specific embodiment, a Therapeutic is indicated for use in treating nerve injury or a nervous system degenerative disorder (see Section 5.8.2) which exhibits *in vitro* promotion of nerve regeneration/neurite extension from nerve cells of the affected patient type.

In addition, administration of an Antagonist Therapeutic of the invention is also indicated in diseases or disorders determined or known to involve a Notch or Serrate dominant activated phenotype ("gain of function" mutations.)

Administration of an Agonist Therapeutic is indicated in diseases or disorders determined or known to involve a Notch or Serrate dominant negative phenotype ("loss of function" mutations). The functions of various structural domains of the Notch protein have been investigated *in vivo*, by ectopically expressing a series of *Drosophila* Notch deletion mutants under the hsp70 heat-shock promoter, as well as eye-specific promoters (see Rebay et al., 1993, Cell 74:319-329). Two classes of dominant phenotypes were observed, one suggestive of Notch loss-of function mutations and the other of Notch gain-of-function mutations. Dominant "activated" phenotypes resulted from overexpression of a protein lacking most extracellular sequences, while dominant "negative" phenotypes resulted from overexpression of a protein lacking most intracellular sequences. The results indicated that Notch functions as a receptor whose extracellular domain mediates ligand-binding, resulting in the transmission of developmental signals by the cytoplasmic domain. We have shown that Serrate binds to the Notch ELR 11 and 12 (see PCT Publication WO 93/12141).

In various specific embodiments, *in vitro* assays can be carried out with representative cells of cell types involved in a patient's disorder, to determine if a Therapeutic has a desired effect upon such cell types.

In another embodiment, cells of a patient tissue sample suspected of being pre-neoplastic are similarly plated out or grown *in vitro*, and exposed to a Therapeutic. The Therapeutic which results in a cell phenotype that is more

normal (i.e., less representative of a pre-neoplastic state, neoplastic state, malignant state, or transformed phenotype) is selected for therapeutic use. Many assays standard in the art can be used to assess whether a pre-neoplastic state, 5 neoplastic state, or a transformed or malignant phenotype, is present. For example, characteristics associated with a transformed phenotype (a set of *in vitro* characteristics associated with a tumorigenic ability *in vivo*) include a more rounded cell morphology, looser substratum attachment, loss 10 of contact inhibition, loss of anchorage dependence, release of proteases such as plasminogen activator, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton surface protein, etc. (see Luria et al., 1978, *General Virology*, 3d 15 Ed., John Wiley & Sons, New York pp. 436-446).

In other specific embodiments, the *in vitro* assays described *supra* can be carried out using a cell line, rather than a cell sample derived from the specific patient to be treated, in which the cell line is derived from or displays 20 characteristic(s) associated with the malignant, neoplastic or pre-neoplastic disorder desired to be treated or prevented, or is derived from the neural or other cell type upon which an effect is desired, according to the present invention.

25 The Antagonist Therapeutics are administered therapeutically (including prophylactically): (1) in diseases or disorders involving increased (relative to normal, or desired) levels of Notch or Serrate function, for example, where the Notch or Serrate protein is overexpressed or 30 overactive; and (2) in diseases or disorders wherein *in vitro* (or *in vivo*) assays indicate the utility of Serrate antagonist administration. The increased levels of Notch or Serrate function can be readily detected by methods such as those described above, by quantifying protein and/or RNA. In 35 *vitro* assays with cells of patient tissue sample or the appropriate cell line or cell type, to determine therapeutic utility, can be carried out as described above.

5.8.1. MALIGNANCIES

Malignant and pre-neoplastic conditions which can be tested as described *supra* for efficacy of intervention with Antagonist or Agonist Therapeutics, and which can be
 5 treated upon thus observing an indication of therapeutic utility, include but are not limited to those described below in Sections 5.8.1 and 5.9.1.

Malignancies and related disorders, cells of which type can be tested *in vitro* (and/or *in vivo*), and upon
 10 observing the appropriate assay result, treated according to the present invention, include but are not limited to those listed in Table 1 (for a review of such disorders, see Fishman et al., 1985, *Medicine*, 2d Ed., J.B. Lippincott Co., Philadelphia):

15

TABLE 1
MALIGNANCIES AND RELATED DISORDERS

20	Leukemia
	acute leukemia
	acute lymphocytic leukemia
	acute myelocytic leukemia
	myeloblastic
	promyelocytic
	myelomonocytic
	monocytic
	erythroleukemia
25	chronic leukemia
	chronic myelocytic (granulocytic) leukemia
	chronic lymphocytic leukemia
	Polycythemia vera
	Lymphoma
	Hodgkin's disease
	non-Hodgkin's disease
30	Multiple myeloma
	Waldenström's macroglobulinemia
	Heavy chain disease
	Solid tumors
	sarcomas and carcinomas
	fibrosarcoma
	myxosarcoma
	liposarcoma
35	chondrosarcoma
	osteogenic sarcoma
	chordoma

angiosarcoma
endotheliosarcoma
lymphangiosarcoma
lymphangioendotheliosarcoma
synovioma
mesothelioma
5 Ewing's tumor
leiomyosarcoma
rhabdomyosarcoma
colon carcinoma
pancreatic cancer
breast cancer
ovarian cancer
10 prostate cancer
squamous cell carcinoma
basal cell carcinoma
adenocarcinoma
sweat gland carcinoma
sebaceous gland carcinoma
papillary carcinoma
papillary adenocarcinomas
15 cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma
hepatoma
bile duct carcinoma
choriocarcinoma
20 seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
testicular tumor
lung carcinoma
small cell lung carcinoma
bladder carcinoma
25 epithelial carcinoma
glioma
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
30 hemangioblastoma
acoustic neuroma
oligodendroglioma
menangioma
melanoma
neuroblastoma
retinoblastoma

35

In specific embodiments, malignancy or dysproliferative changes (such as metaplasias and dysplasias) are treated or prevented in epithelial tissues such as those in the cervix, esophagus, and lung.

5 Malignancies of the colon and cervix exhibit increased expression of human Notch relative to such non-malignant tissue (see PCT Publication no. WO 94/07474 published April 14, 1994, incorporated by reference herein in its entirety). Thus, in specific embodiments, malignancies
10 or premalignant changes of the colon or cervix are treated or prevented by administering an effective amount of an Antagonist Therapeutic, e.g., a Serrate derivative, that antagonizes Notch function. The presence of increased Notch expression in colon, and cervical cancer suggests that many
15 more cancerous and hyperproliferative conditions exhibit upregulated Notch. Thus, in specific embodiments, various cancers, e.g., breast cancer, squamous adenocarcinoma, seminoma, melanoma, and lung cancer, and premalignant changes therein, as well as other hyperproliferative disorders, can
20 be treated or prevented by administration of an Antagonist Therapeutic that antagonizes Notch function.

5.8.2. NERVOUS SYSTEM DISORDERS

Nervous system disorders, involving cell types
25 which can be tested as described supra for efficacy of intervention with Antagonist or Agonist Therapeutics, and which can be treated upon thus observing an indication of therapeutic utility, include but are not limited to nervous system injuries, and diseases or disorders which result in
30 either a disconnection of axons, a diminution or degeneration of neurons, or demyelination. Nervous system lesions which may be treated in a patient (including human and non-human mammalian patients) according to the invention include but are not limited to the following lesions of either the
35 central (including spinal cord, brain) or peripheral nervous systems:

- 5 (i) traumatic lesions, including lesions caused by physical injury or associated with surgery, for example, lesions which sever a portion of the nervous system, or compression injuries;
- 10 (ii) ischemic lesions, in which a lack of oxygen in a portion of the nervous system results in neuronal injury or death, including cerebral infarction or ischemia, or spinal cord infarction or ischemia;
- 15 (iii) malignant lesions, in which a portion of the nervous system is destroyed or injured by malignant tissue which is either a nervous system associated malignancy or a malignancy derived from non-nervous system tissue;
- 20 (iv) infectious lesions, in which a portion of the nervous system is destroyed or injured as a result of infection, for example, by an abscess or associated with infection by human immunodeficiency virus, herpes zoster, or herpes simplex virus or with Lyme disease, tuberculosis, syphilis;
- 25 (v) degenerative lesions, in which a portion of the nervous system is destroyed or injured as a result of a degenerative process including but not limited to degeneration associated with Parkinson's disease, Alzheimer's disease, Huntington's chorea, or amyotrophic lateral sclerosis;
- 30 (vi) lesions associated with nutritional diseases or disorders, in which a portion of the nervous system is destroyed or injured by a nutritional disorder or disorder of metabolism including but not limited to, vitamin B12 deficiency, folic acid deficiency, Wernicke disease, tobacco-alcohol amblyopia, Marchiafava-Bignami disease (primary
- 35

- degeneration of the corpus callosum), and alcoholic cerebellar degeneration;
- (vii) neurological lesions associated with systemic diseases including but not limited to diabetes (diabetic neuropathy, Bell's palsy), systemic lupus erythematosus, carcinoma, or sarcoidosis;
- (viii) lesions caused by toxic substances including alcohol, lead, or particular neurotoxins; and
- (ix) demyelinated lesions in which a portion of the nervous system is destroyed or injured by a demyelinating disease including but not limited to multiple sclerosis, human immunodeficiency virus-associated myelopathy, transverse myelopathy or various etiologies, progressive multifocal leukoencephalopathy, and central pontine myelinolysis.

Therapeutics which are useful according to the invention for treatment of a nervous system disorder may be selected by testing for biological activity in promoting the survival or differentiation of neurons (see also Section 5.8). For example, and not by way of limitation, Therapeutics which elicit any of the following effects may be useful according to the invention:

- (i) increased survival time of neurons in culture;
- (ii) increased sprouting of neurons in culture or *in vivo*;
- (iii) increased production of a neuron-associated molecule in culture or *in vivo*, e.g., choline acetyltransferase or acetylcholinesterase with respect to motor neurons; or
- (iv) decreased symptoms of neuron dysfunction in *vivo*.

Such effects may be measured by any method known in the art. In preferred, non-limiting embodiments, increased survival of neurons may be measured by the method set forth in Arakawa et al. (1990, J. Neurosci. 10:3507-3515); increased sprouting of

neurons may be detected by methods set forth in Pestronk et al. (1980, Exp. Neurol. 70:65-82) or Brown et al. (1981, Ann. Rev. Neurosci. 4:17-42); increased production of neuron-associated molecules may be measured by bioassay, enzymatic
5 assay, antibody binding, Northern blot assay, etc., depending on the molecule to be measured; and motor neuron dysfunction may be measured by assessing the physical manifestation of motor neuron disorder, e.g., weakness, motor neuron conduction velocity, or functional disability.

10 In a specific embodiment, motor neuron disorders that may be treated according to the invention include but are not limited to disorders such as infarction, infection, exposure to toxin, trauma, surgical damage, degenerative disease or malignancy that may affect motor neurons as well
15 as other components of the nervous system, as well as disorders that selectively affect neurons such as amyotrophic lateral sclerosis, and including but not limited to progressive spinal muscular atrophy, progressive bulbar palsy, primary lateral sclerosis, infantile and juvenile
20 muscular atrophy, progressive bulbar paralysis of childhood (Fazio-Londe syndrome), poliomyelitis and the post polio syndrome, and Hereditary Motorsensory Neuropathy (Charcot-Marie-Tooth Disease).

25 5.8.3. TISSUE REPAIR AND REGENERATION

In another embodiment of the invention, a Therapeutic of the invention is used for promotion of tissue regeneration and repair, including but not limited to treatment of benign dysproliferative disorders. Specific
30 embodiments are directed to treatment of cirrhosis of the liver (a condition in which scarring has overtaken normal liver regeneration processes), treatment of keloid (hypertrophic scar) formation (disfiguring of the skin in which the scarring process interferes with normal renewal),
35 psoriasis (a common skin condition characterized by excessive proliferation of the skin and delay in proper cell fate determination), and baldness (a condition in which terminally

differentiated hair follicles (a tissue rich in Notch) fail to function properly). In another embodiment, a Therapeutic of the invention is used to treat degenerative or traumatic disorders of the sensory epithelium of the inner ear.

5

5.9. PROPHYLACTIC USES

5.9.1. MALIGNANCIES

The Therapeutics of the invention can be administered to prevent progression to a neoplastic or malignant state, including but not limited to those disorders listed in Table 1. Such administration is indicated where the Therapeutic is shown in assays, as described *supra*, to have utility for treatment or prevention of such disorder. Such prophylactic use is indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred (for review of such abnormal growth conditions, see Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-79.) Hyperplasia is a form of controlled cell proliferation involving an increase in cell number in a tissue or organ, without significant alteration in structure or function. As but one example, endometrial hyperplasia often precedes endometrial cancer. Metaplasia is a form of controlled cell growth in which one type of adult or fully differentiated cell substitutes for another type of adult cell. Metaplasia can occur in epithelial or connective tissue cells. Atypical metaplasia involves a somewhat disorderly metaplastic epithelium. Dysplasia is frequently a forerunner of cancer, and is found mainly in the epithelia; it is the most disorderly form of non-neoplastic cell growth, involving a loss in individual cell uniformity and in the architectural orientation of cells. Dysplastic cells often have abnormally large, deeply stained nuclei, and exhibit pleomorphism. Dysplasia characteristically occurs where there exists chronic

irritation or inflammation, and is often found in the cervix, respiratory passages, oral cavity, and gall bladder.

Alternatively or in addition to the presence of abnormal cell growth characterized as hyperplasia, 5 metaplasia, or dysplasia, the presence of one or more characteristics of a transformed phenotype, or of a malignant phenotype, displayed *in vivo* or displayed *in vitro* by a cell sample from a patient, can indicate the desirability of prophylactic/therapeutic administration of a Therapeutic of 10 the invention. As mentioned *supra*, such characteristics of a transformed phenotype include morphology changes, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, expression of fetal 15 antigens, disappearance of the 250,000 dalton cell surface protein, etc. (see also *id.*, at pp. 84-90 for characteristics associated with a transformed or malignant phenotype).

In a specific embodiment, leukoplakia, a benign-appearing hyperplastic or dysplastic lesion of the 20 epithelium, or Bowen's disease, a carcinoma *in situ*, are pre-neoplastic lesions indicative of the desirability of prophylactic intervention.

In another embodiment, fibrocystic disease (cystic hyperplasia, mammary dysplasia, particularly adenosis (benign 25 epithelial hyperplasia)) is indicative of the desirability of prophylactic intervention.

In other embodiments, a patient which exhibits one or more of the following predisposing factors for malignancy is treated by administration of an effective amount of a 30 Therapeutic: a chromosomal translocation associated with a malignancy (e.g., the Philadelphia chromosome for chronic myelogenous leukemia, t(14;18) for follicular lymphoma, etc.), familial polyposis or Gardner's syndrome (possible forerunners of colon cancer), benign monoclonal gammopathy (a 35 possible forerunner of multiple myeloma), and a first degree kinship with persons having a cancer or precancerous disease showing a Mendelian (genetic) inheritance pattern (e.g.,

familial polyposis of the colon, Gardner's syndrome, hereditary exostosis, polyendocrine adenomatosis, medullary thyroid carcinoma with amyloid production and pheochromocytoma, Peutz-Jeghers syndrome, neurofibromatosis of Von Recklinghausen, retinoblastoma, carotid body tumor, cutaneous melanocarcinoma, intraocular melanocarcinoma, xeroderma pigmentosum, ataxia telangiectasia, Chediak-Higashi syndrome, albinism, Fanconi's aplastic anemia, and Bloom's syndrome; see Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 112-113) etc.)

In another specific embodiment, an Antagonist Therapeutic of the invention is administered to a human patient to prevent progression to breast, colon, or cervical cancer.

15

5.9.2. OTHER DISORDERS

In other embodiments, a Therapeutic of the invention can be administered to prevent a nervous system disorder described in Section 5.8.2, or other disorder (e.g., liver cirrhosis, psoriasis, keloids, baldness) described in Section 5.8.3.

5.10. DEMONSTRATION OF THERAPEUTIC OR PROPHYLACTIC UTILITY

The Therapeutics of the invention can be tested *in vivo* for the desired therapeutic or prophylactic activity. For example, such compounds can be tested in suitable animal model systems prior to testing in humans, including but not limited to rats, mice, chicken, cows, monkeys, rabbits, etc. For *in vivo* testing, prior to administration to humans, any animal model system known in the art may be used.

5.11. ANTISENSE REGULATION OF SERRATE EXPRESSION

The present invention provides the therapeutic or prophylactic use of nucleic acids of at least six or of at least ten nucleotides that are antisense to a gene or cDNA encoding a vertebrate Serrate or a portion thereof.

"Antisense" as used herein refers to a nucleic acid capable of hybridizing to a portion of a vertebrate *Serrate* RNA (preferably mRNA) by virtue of some sequence complementarity. Such antisense nucleic acids have utility as Antagonist
5 Therapeutics of the invention, and can be used in the treatment or prevention of disorders as described *supra* in Section 5.8 and its subsections.

The antisense nucleic acids of the invention can be oligonucleotides that are double-stranded or single-stranded,
10 RNA or DNA or a modification or derivative thereof, which can be directly administered to a cell, or which can be produced intracellularly by transcription of exogenous, introduced sequences.

In a specific embodiment, the *Serrate* antisense
15 nucleic acids provided by the instant invention can be used for the treatment of tumors or other disorders, the cells of which tumor type or disorder can be demonstrated (*in vitro* or *in vivo*) to express a *Serrate* gene or a *Notch* gene. Such demonstration can be by detection of RNA or of protein.

20 The invention further provides pharmaceutical compositions comprising an effective amount of the *Serrate* antisense nucleic acids of the invention in a pharmaceutically acceptable carrier, as described *infra* in Section 5.12. Methods for treatment and prevention of
25 disorders (such as those described in Sections 5.8 and 5.9) comprising administering the pharmaceutical compositions of the invention are also provided.

In another embodiment, the invention is directed to methods for inhibiting the expression of a *Serrate* nucleic
30 acid sequence in a prokaryotic or eukaryotic cell comprising providing the cell with an effective amount of a composition comprising an antisense vertebrate *Serrate* nucleic acid of the invention.

Serrate antisense nucleic acids and their uses are
35 described in detail below.

5.11.1. VERTEBRATE SERRATE ANTISENSE NUCLEIC ACIDS

The vertebrate Serrate antisense nucleic acids are of at least six nucleotides and are preferably oligonucleotides (ranging preferably from 10 to about 50 oligonucleotides). In specific aspects, the oligonucleotide contains at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides antisense to a Serrate gene. The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone. The oligonucleotide may include other appending groups such as peptides, or agents facilitating transport across the cell membrane (see, e.g., Letsinger et al., 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556; Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652; PCT Publication No. WO 88/09810, published December 15, 1988) or blood-brain barrier (see, e.g., PCT Publication No. WO 89/10134, published April 25, 1988), hybridization-triggered cleavage agents (see, e.g., Krol et al., 1988, BioTechniques 6:958-976) or intercalating agents (see, e.g., Zon, 1988, Pharm. Res. 5:539-549).

In a preferred aspect of the invention, a vertebrate Serrate antisense oligonucleotide is provided, preferably of single-stranded DNA. In a most preferred aspect, such an oligonucleotide comprises a sequence antisense to the sequence encoding an SH3 binding domain or a Notch-binding domain of Serrate, most preferably, of a human Serrate homolog. The oligonucleotide may be modified at any position on its structure with substituents generally known in the art.

The Serrate antisense oligonucleotide may comprise at least one modified base moiety which is selected from the group including but not limited to 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxymethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine,

5-carboxymethylaminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil, (acp3)w, and 2,6-diaminopurine.

15 In another embodiment, the oligonucleotide comprises at least one modified sugar moiety selected from the group including but not limited to arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide 20 comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal or analog thereof.

25 In yet another embodiment, the oligonucleotide is an α -anomeric oligonucleotide. An α -anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β -units, the strands run parallel to each other (Gautier et al., 1987, Nucl. Acids 30 Res. 15:6625-6641).

The oligonucleotide may be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, hybridization-triggered cleavage agent, etc.

35 Oligonucleotides of the invention may be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially

available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides may be synthesized by the method of Stein et al. (1988, Nucl. Acids Res. 16:3209), methylphosphonate oligonucleotides can be
5 prepared by use of controlled pore glass polymer supports (Sarin et al., 1988, Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

In a specific embodiment, the Serrate antisense oligonucleotide comprises catalytic RNA, or a ribozyme (see,
10 e.g., PCT International Publication WO 90/11364, published October 4, 1990; Sarver et al., 1990, Science 247:1222-1225). In another embodiment, the oligonucleotide is a 2'-O-methylribonucleotide (Inoue et al., 1987, Nucl. Acids Res. 15:6131-6148), or a chimeric RNA-DNA analogue (Inoue et al.,
15 1987, FEBS Lett. 215:327-330).

In an alternative embodiment, the Serrate antisense nucleic acid of the invention is produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by
20 a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector would contain a sequence encoding the Serrate antisense nucleic acid. Such a vector can remain episomal or become chromosomally integrated, as long as it
25 can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence
30 encoding the Serrate antisense RNA can be by any promoter known in the art to act in mammalian, preferably human, cells. Such promoters can be inducible or constitutive. Such promoters include but are not limited to: the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-
35 310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al.,

1981, Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445), the regulatory sequences of the metallothionein gene (Brinster et al., 1982, Nature 296:39-42), tc.

The antisense nucleic acids of the invention
5 comprise a sequence complementary to at least a portion of an RNA transcript specific to a vertebrate Serrate gene, preferably a human Serrate gene. However, absolute complementarity, although preferred, is not required. A sequence "complementary to at least a portion of an RNA," as
10 referred to herein, means a sequence having sufficient complementarity to be able to hybridize with the RNA, forming a stable duplex; in the case of double-stranded Serrate antisense nucleic acids, a single strand of the duplex DNA may thus be tested, or triplex formation may be assayed. The
15 ability to hybridize will depend on both the degree of complementarity and the length of the antisense nucleic acid. Generally, the longer the hybridizing nucleic acid, the more base mismatches with a Serrate RNA it may contain and still form a stable duplex (or triplex, as the case may be). One
20 skilled in the art can ascertain a tolerable degree of mismatch by use of standard procedures to determine the melting point of the hybridized complex.

5.11.2. THERAPEUTIC UTILITY OF VERTEBRATE 25 SERRATE ANTISENSE NUCLEIC ACIDS

The vertebrate Serrate antisense nucleic acids can be used to treat (or prevent) malignancies or other disorders, of a cell type which has been shown to express Serrate or Notch. In specific embodiments, the malignancy is
30 cervical, breast, or colon cancer, or squamous adenocarcinoma. Malignant, neoplastic, and pre-neoplastic cells which can be tested for such expression include but are not limited to those described *supra* in Sections 5.8.1 and 5.9.1. In a preferred embodiment, a single-stranded DNA
35 antisense Serrate oligonucleotide is used.

Malignant (particularly, tumor) cell types which express Serrate or Notch RNA can be identified by various

methods known in the art. Such methods include but are not limited to hybridization with a Serrate or Notch-specific nucleic acid (e.g. by Northern hybridization, dot blot hybridization, *in situ* hybridization), observing the ability of RNA from the cell type to be translated *in vitro* into Notch or Serrate, immunoassay, etc. In a preferred aspect, primary tumor tissue from a patient can be assayed for Notch or Serrate expression prior to treatment, e.g., by immunocytochemistry or *in situ* hybridization.

10 Pharmaceutical compositions of the invention (see Section 5.12), comprising an effective amount of a vertebrate Serrate antisense nucleic acid in a pharmaceutically acceptable carrier, can be administered to a patient having a malignancy which is of a type that expresses Notch or Serrate
15 RNA or protein.

The amount of Serrate antisense nucleic acid which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical
20 techniques. Where possible, it is desirable to determine the antisense cytotoxicity of the tumor type to be treated *in vitro*, and then in useful animal model systems prior to testing and use in humans.

In a specific embodiment, pharmaceutical
25 compositions comprising vertebrate Serrate antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the Serrate antisense nucleic acids. In a
30 specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable tumor antigens (Leonetti et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2448-2451; Renneisen et al., 1990, J. Biol. Chem. 265:16337-16342).

35

5.12. THERAPEUTIC/PROPHYLACTIC
ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to animals such as cows, pigs, chickens, etc., and is preferably a mammal, and most preferably human.

Various delivery systems are known and can be used to administer a Therapeutic of the invention, e.g., encapsulation in liposomes, microparticles, microcapsules, expression by recombinant cells, receptor-mediated endocytosis (see, e.g., Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a Therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (e.g., oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, e.g., by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be

achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, e.g., in conjunction with a wound dressing after surgery, by injection, by means of a catheter, by means of a suppository, 5 or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers. In one embodiment, administration can be by direct injection at the site (or former site) of a malignant tumor or neoplastic or pre- 10 neoplastic tissue.

In another embodiment, the Therapeutic can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein 15 and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, *ibid.*, pp. 317-327; see generally *ibid.*)

In yet another embodiment, the Therapeutic can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, CRC Crit. Ref. 20 Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled 25 Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 30 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, *supra*, vol. 2, pp. 35 115-138 (1984)).

Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

In a specific embodiment where the Therapeutic is a nucleic acid encoding a protein Therapeutic, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g., by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox-like peptide which is known to enter the nucleus (see e.g., Joliot et al., 1991, Proc. Natl. Acad. Sci. USA 88:1864-1868), etc. Alternatively, a nucleic acid Therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

In specific embodiments directed to treatment or prevention of particular disorders, preferably the following forms of administration are used:

20	<u>Disorder</u>	<u>Preferred Forms of Administration</u>
	Cervical cancer	Topical
	Gastrointestinal cancer	Oral; intravenous
	Lung cancer	Inhaled; intravenous
25	Leukemia	Intravenous; extracorporeal
	Metastatic carcinomas	Intravenous; oral
	Brain cancer	Targeted; intravenous; intrathecal
	Liver cirrhosis	Oral; intravenous
	Psoriasis	Topical
30	Keloids	Topical
	Baldness	Topical
	Spinal cord injury	Targeted; intravenous; intrathecal
	Parkinson's disease	Targeted; intravenous; intrathecal
	Motor neuron disease	Targeted; intravenous; intrathecal
35	Alzheimer's disease	Targeted; intravenous; intrathecal

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term

5 "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or

10 vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier

15 when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose,

20 sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying

25 agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides.

30 Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W.

35 Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to

provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The Therapeutics of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation

will also depend on the route of administration, and the seriousness of the disease or disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. However, suitable dosage ranges for 5 intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose- 10 response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

15 The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental 20 agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

25 5.13. DIAGNOSTIC UTILITY

Vertebrate Serrate proteins, analogues, derivatives, and subsequences thereof, vertebrate Serrate nucleic acids (and sequences complementary thereto), anti-vertebrate Serrate antibodies, have uses in diagnostics. 30 Such molecules can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders affecting Serrate expression, or monitor the treatment thereof. In particular, such an immunoassay is carried out by a method comprising contacting 35 a sample derived from a patient with an anti-Serrate antibody under conditions such that immunospecific binding can occur, and detecting or measuring the amount of any immunospecific

binding by the antibody. In a specific aspect, such binding of antibody, in tissue sections, preferably in conjunction with binding of anti-Notch antibody can be used to detect aberrant Notch and/or Serrate localization or aberrant levels of Notch-Serrate colocalization in a disease state. In a specific embodiment, antibody to Serrate can be used to assay in a patient tissue or serum sample for the presence of Serrate where an aberrant level of Serrate is an indication of a diseased condition. Aberrant levels of Serrate binding ability in an endogenous Notch protein, or aberrant levels of binding ability to Notch (or other Serrate ligand) in an endogenous Serrate protein may be indicative of a disorder of cell fate (e.g., cancer, etc.) By "aberrant levels," is meant increased or decreased levels relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disorder.

The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few.

Vertebrate Serrate genes and related nucleic acid sequences and subsequences, including complementary sequences, and other toporythmic gene sequences, can also be used in hybridization assays. Vertebrate Serrate nucleic acid sequences, or subsequences thereof comprising about at least 8 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant changes in Serrate expression and/or activity as described *supra*. In particular, such a hybridization assay is carried out by a method comprising

contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to Serrate DNA or RNA, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.

- 5 Additionally, since Serrate binds to Notch, vertebrate Serrate or a binding portion thereof can be used to assay for the presence and/or amounts of Notch in a sample, e.g., in screening for malignancies which exhibit increased Notch expression such as colon and cervical
10 cancers.

6. ISOLATION AND CHARACTERIZATION
OF A MOUSE SERRATE HOMOLOG

A mouse Serrate homolog, termed M-Serrate-1, was
15 isolated as follows:

Mouse Serrate-1 gene

Tissue origin: 10.5-day mouse embryonic RNA

Isolation method:

- a) random primed cDNA against above RNA
b) PCR of above cDNA using
20 PCR primer 1: CGI(C/T)TTTGC(C/T)TIAA(A/G)(G/C)AITA(C/T)CA
(SEQ ID NO: 9) {encoding RLCKK(H/E)YQ (SEQ ID NO:10)}:
PCR primer 2: TCIATGCAIGTICCC(A/G)TT (SEQ ID NO:11)
{encoding NGGTCID (SEQ ID NO:12)}
25 Amplification conditions: 50 ng cDNA, 1 µg each primer,
0.2 mM dNTP's, 1.8 U Taq (Perkin-Elmer) in 50 µl of supplied
buffer, 40 cycles of: 94°C/30 sec, 45°C/2 min, 72°C/1 min
extended by 2 sec each cycle.

- 30 Yielded a 1.8 kb fragment which was sequenced at both ends
and identified as corresponding to C-Serrate-1

Partial DNA sequence of M-Serrate-1:

From 5' end:

- 35 GTCCCGCGTCACTGCCGGGGGACCCTGCAGCTTCGGCTCAGGGTCTACGCCTGTCATCGGG
GGTAACACCTTCAATCTCAAGGCCAGCCGTGGCAACGACCGTAATCGCATCGTACTGCCTT
TCAGTTTCACCTGGCCGAGGTCCTACACTTTGCTGGTGGAG (SEQ ID NO:13)

Protein translation of above:

SRVTAGGPCSFGSGSTPVIGGNTFNLKASRGNDRNRIVLPFSFTWPRSYTLLVE
(SEQ ID NO:14) (corresponds to amino-terminal sequence
upstream of the DSL domain)

5

From 3' end (but coding strand)

TCTTCTAACGTCTGTGGTCCCCATGGCAAGTGCAAGAGCCAGTCGGCAGGCAAATTCACCT
GTGACTGTAACAAAGGCTTCACCGGCACCTACTGCCATGAAAATATCAACGACTGCGAGAG
CAACCCCTGTAAA (SEQ ID NO:15)

10 Protein translation of above:

SSNVCGPHGKCKSQSAGKFTCDCNKGFTGTYPHENINDCESNPCK (SEQ ID NO:16)
(within tandemly arranged EGF-like repeats)

Expression pattern: The expression pattern was determined to
15 be the same as that observed for *C-Serrate-1* (chicken
Serrate) (see Section 11 *infra*), including expression in the
developing central nervous system, peripheral nervous system,
limb, kidney, lens, and vascular system.

20

7. ISOLATION AND CHARACTERIZATION OF A *XENOPUS* SERRATE HOMOLOG

A *Xenopus* Serrate homolog, termed *Xenopus Serrate-1*
was isolated as follows:

***Xenopus Serrate-1* gene**

25 Tissue origin: neurula-stage embryonic RNA

Isolation method:

a) random primed cDNA against above RNA

b) PCR using:

Primer 1: CGI(C/T)TTTGC(C/T)TIAA(A/G)(G/C)AITA(C/T)CA
30 (SEQ ID NO:9) {encoding RLCKK(H/E)YQ (SEQ ID NO:10)}:

PCR primer 2: TCIATGCAIGTICICCA(A/G)TT (SEQ ID NO:11)
{encoding NGGTCID (SEQ ID NO:12)}

Amplification conditions: 50 ng cDNA, 1 µg each primer,
0.2 mM dNTP's, 1.8 U Taq (Perkin-Elmer) in 50 µl of supplied
35 buffer. 40 cycles of: 94°C/30 sec, 45°C/2 min, 72°C/1 min
extend d by 2 sec each cycle.

Yielded a ~700 bp fragment which was partially sequenced to confirm its relationship to *C-Serrate-1*.

8. ISOLATION AND CHARACTERIZATION OF A CHICK SERRATE HOMOLOG

5 In the example herein, we report the cloning and sequence of a chick Serrate homolog, *C-Serrate*, and of fragments of two chick Notch homologs, *C-Notch-1* and *C-Notch-2*, together with their expression patterns during
10 early embryogenesis. The patterns of transcription of *C-Serrate* overlaps with that of *C-Notch-1* in many regions of the embryo, suggesting that *C-Notch-1*, like Notch in *Drosophila*, is a receptor for Serrate. In particular, Notch and Serrate are expressed in the neurogenic regions of the
15 developing central and peripheral nervous system.

Our data show that Serrate, a known ligand of Notch, has been conserved from arthropods to chordates. The overlapping expression patterns suggest conservation of its functional relationship with Notch and imply that development
20 of the chick and in particular of its central nervous system involves the interaction of *C-Notch-1* with Serrate at several specific locations.

Materials and Methods

25 *Embryos*

White Leghorn chicken eggs were obtained from University Park Farm and incubated at 38°C. Embryos were staged according to Hamburger and Hamilton (1951, J. Exp. Zool. 88:49-92).

30 *Cloning of chicken homologs of Notch*

Approximately 1000 base pair PCR fragments of the chicken *Notch 1* and *Notch 2* genes were amplified from otic explant RNA (see below) using degenerate primers and PCR
35 conditions as outlined in Lardelli and Lendahl (1993, Exp. Cell Res. 204:364-372). The PCR fragment was subcloned into Bluescript KS-, sequenced and used as a template for making a

DIG antisense RNA probe (RNA Transcription Kit, Stratagene; DIG RNA labelling mix, Boehringer Mannheim).

Cloning of a chicken homologue of Drosophila Serrate

5 Otic explants were dissected from embryos of stages 8 to 13. Each otic explant consisted of the two otic cups, a short section of intervening hindbrain and pharynx and the associated head ectoderm and mesenchyme. RNA was extracted using a modification of standard protocols (Sambrook et al., 10 1989, in *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York) and polyA⁺ mRNA was isolated from total RNA using the PolyATtract mRNA Isolation System (Promega). First strand cDNA was synthesized using the SuperScript Preamplification 15 System (Gibco).

 PCR and degenerate primers were used to amplify a fragment of a chicken gene homologous to the *Drosophila* gene *Serrate* from the otic explant cDNA. The primers were designed to recognize peptide motifs found in both the fly 20 *Delta* and *Serrate* proteins:

- 1) primer 1, 5-CGI(T/C)TITGC(T/C)TIAA(G/A)(G/C)AITA(C/T)CA-3' (SEQ ID NO:17), corresponds to the motif RLCLK(E/H)YQ (SEQ ID NO:18) located at the amino-terminus of the fly *Delta* and *Serrate* proteins.
- 25 2) primer 2, 5'-TCIATGCAIGTICCC(A/G)TT-3' (SEQ ID NO:11), corresponds to the motif NGGTCID (SEQ ID NO:12) found in several of the EGF-like repeats. The PCR conditions were as follows: 35 cycles of 94°C for 1 minute, 45°C for 1.5 minutes and 72°C for 2 minutes; followed by a final extension step of 30 72°C for 10 minutes. A PCR product of approximately 900 base pairs in length was purified, subcloned into Bluescript KS- (Stratagene) and its DNA sequence partially determined to confirm that it was a likely *Serrate* homolog. It was then used to recover larger cDNA clones by screening two cDNA 35 libraries:

- 1) a stage 8-13 otic explant random primed cDNA library

2) a stage 17 chick spinal cord oligo dT primed cDNA library
Overlapping cDNAs were isolated, and two (termed 9 and 3A.1)
that together cover almost the entire coding region of the
gene were subcloned into Bluescript KS-. DNA sequence was
5 determined from nested deletion series generated using the
double-stranded Nested Deletion Kit (Pharmacia) and Sanger
dideoxy chain termination method with the Sequenase enzyme
(US Biochemical Corporation). Sequences were aligned and
analyzed using Geneworks 2.3 and Intelligenetics. Homology
10 searches were done using the program Sharq.

To obtain the most 5' end of the open reading
frame, a number of other PCR based strategies were used
including the screening of a number of other libraries (cDNA
and genomic) using the method of Lardelli et al. (1994,
15 Mechanisms of Development 46:123-136).

In situ hybridization

Patterns of gene transcription were determined by
in situ hybridization using DIG-labeled RNA probes and:
20 1) a high-stringency wholemount *in situ* hybridization
protocol, and
2) *in situ* hybridization on cryostat sections based on the
protocol of Strähle et al. (1994, Trends in Genet. 10:7).

25 Results

To obtain insight into the likely role of chick
Serrate in the vertebrate embryo, we examined its expression
in relation to that of chick Notch, since functional coupling
of Notch and Serrate occurs in *Drosophila*. Two chick Notch
30 homologs were obtained as described below.

**C-Notch-1 and C-Notch-2 are apparent counterparts of the
rodent Notch-1 and Notch-2 genes, respectively**

We searched for Notch homologs in the chick by PCR,
35 using cDNA prepared from two-day chick embryos and degenerate
primers based on conserved regions common to the known rodent
Notch homologs. In this way, we obtained fragments, each

approximately 1000 nucleotides long, of two distinct genes, which we have called C-Notch-1 and C-Notch-2. The fragments extend from the third Notch/lin12 repeat up to and including the last five or so EGF-like repeats. EGF-like repeats are present in a large number of proteins, most of which are otherwise unrelated to Notch. The three Notch/lin12 repeats, however, are peculiar to the Notch family of genes and are found in all its known members. C-Notch-1 shows the highest degree of amino-acid identity with rodent Notch1 (Weinmaster et al., 1991, Development 113:199-205), and is expressed in broadly similar domains to rodent Notch1 (see below). Of the rodent Notch genes, C-Notch-2 appears most similar to Notch2 (Weinmaster et al., 1992, Development 116:931-941).

We examined the expression patterns of C-Notch-1 in early embryos by *in situ* hybridization. C-Notch-1 was expressed in the 1- to 2-day chick embryo in many well-defined domains, including the neural tube, the presomitic mesoderm, the nephrogenic mesoderm (the prospective mesonephros), the nasal placode, the otic placode/vesicle, the lens placode, the epibranchial placodes, the endothelial lining of the vascular system, in the heart, and the apical ectodermal ridges (AER) of the limb buds. These sites match the reported sites of Notch1 expression in rodents at equivalent stages (Table II). Taking the sequence data together with the expression data, we conclude that C-Notch-1 is either the chick ortholog of rodent Notch1, or a very close relative of it.

Table II

COMPARISON OF DOMAINS OF RODENT-NOTCH1
AND CHICK NOTCH-1 EXPRESSION THROUGHOUT EMBRYOGENESIS

Body Region	R-Notch1 ^a	C-Notch1
primitive streak	+	+
Hensen's node	-	-
neural tube	+	+

	retina	+	+
	lens	+	+
	otic placode/vesicle	+	+
5	epibranchial placodes	+	+
	nasal placode	+	+
	dorsal root ganglia	+	+
	presomitic mesoderm	+	+
	somites	+	+
10	notochord	?	+
	mesonephric kidney	+	+
	metanephric kidney	+	+
	blood vessels	+	+
	heart	+	+
15	whisker follicles	+	N/A
	thymus	+	?
	toothbuds	+	N/A
	salivary gland	+	?
20	limb bud (AER)	?	+
	* from Weinmaster et al., 1991, Development 113:199-205; Franco del Amo et al., 1992, Development 115:737-744; Reaume et al., 1992, Dev. Biol. 154:377-387; Kopan and Weintraub, 1993, J. Cell. Biol. 121:631-641; Lardelli et al., 1994, Mech. of Dev. 46:123-126.		

25

C-Serrate is a homolog of *Drosophila* Serrate, and codes for a candidate ligand for a receptor belonging to the Notch family

In *Drosophila*, two ligands for Notch are known, encoded by the two related genes *Delta* and *Serrate*. The amino-acid sequences corresponding to these genes are homologous at their 5' ends, including a region, the DSL motif, which is necessary and sufficient for in vitro binding to Notch. To isolate a fragment of a chicken homolog of *Serrate*, we used PCR and degenerate primers designed to recognize sequences on either side of the DSL motif (see Materials and methods). A 900 base pair PCR fragment was

recovered and used to screen a library, allowing us to isolate overlapping cDNA clones. The DNA sequence of the cDNA clones revealed an almost complete single open reading frame of 3582 nucleotides, lacking only a few 5' bases.

- 5 Comparison with the amino acid sequences of *Drosophila* Delta and Serrate suggests that we are missing only the portion of the coding sequence that encodes part of the signal sequence of the chick Serrate protein.

Translation of the nucleotide sequence

- 10 (SEQ ID NO:5) (Fig. 3) predicts a protein of 1230 amino acids (SEQ ID NO:6) (Fig. 4). A hydropathy plot reveals a single hydrophobic region characteristic of a transmembrane domain (Kyte and Doolittle, 1982, J. Mol. Biol. 157:105-132). In addition, the protein has sixteen EGF-like repeats organized
15 in a tandem array in its extracellular domain. Comparison of the chick sequence with sequences of *D. melanogaster* Delta and Serrate suggests that the clones encode a chicken homolog of Serrate (Fig. 5; Fig. 6). Whereas *Drosophila* Serrate contains 14 EGF-like repeats with large insertions in repeats
20 4, 6 and 10, the chicken homolog has an extra two EGF-like repeats and only one small insertion of 16 amino acids in the 10th repeat. Both proteins have a second cysteine-rich region between the EGF-like repeats and the transmembrane domain; the spacing of the cysteines in this region is almost
25 identical in the two proteins (compare CX₂CXCX₆CX₄CX₁CX₅CX₇CX₄CX₅C in *Drosophila* Serrate with CX₂CXCX₆CX₄CX₅CX₇CX₄CX₅C in C-Serrate). The intracellular domain of C-Serrate bears no significant homology to the intracellular domains of either *Drosophila* Delta or Serrate.

30

C-Serrate is expressed in the central nervous system, cranial placodes, nephric mesoderm, vascular system, and limb bud mesenchyme

- 35 *In situ* hybridization was performed to examine the expression of C-Serrate in whole-mount preparations during early embryogenesis, from stage 4 to stage 21, at intervals

of roughly 12 hours. Later stages were studied by *in situ* hybridization on cryosections.

The main sites of early expression of C-Serrate, as seen in whole mounts, can be grouped under five headings:
5 central nervous system, cranial placodes, nephric mesoderm, vascular system, and limb bud mesenchyme.

Central nervous system

The first detectable expression of C-Serrate was
10 seen in the central nervous system at stage 6 (0 somites/24 hrs), within the posterior portion of the neural plate. By stage 10 (9-11 somites/35.5 hrs), a strong stripe of expression was seen in the prospective diencephalon. Additional faint staining was seen in the hindbrain and in
15 the prospective spinal cord.

At stage 13, there were several patches of expression in the neural tube. In the diencephalon, there was a strong triangular stripe of expression that appeared to correspond to neuromere D2. There were two patches (one on
20 either side of the midline) on the floor of the anterior mesencephalon as well as diffuse staining in the dorsal mesencephalon. In the hindbrain and rostral spinal cord, there were two longitudinal stripes of expression on either side of the midline: one along the dorsal edge of the neural
25 tube and a second more ventral one, adjacent to the floor plate. Both were located within the domain of (rat) *Notch 1* expression. The anterior limit of the ventral stripe was at the midbrain/hindbrain boundary. The dorsal stripe was continuous with the expression in the dorsal mesencephalon.
30 In the anterior spinal cord, expression was more spotty, the stripes being replaced by isolated scattered cells expressing C-Serrate.

At stage 17 (58 hrs), expression in the diencephalon and midbrain was unchanged. In the hindbrain
35 and spinal cord, there were an additional two longitudinal stripes: one midway along the dorsoventral axis and a second wider more ventral stripe; the anterior limits of these

stripes coincided with the anterior border of rhombomere 2. All four longitudinal stripes in the hindbrain continued into the spinal cord of the embryo; decreasing towards its posterior end. These stripes of expression were maintained at least up to and including stage 31 (E7). By stage 21 (84 hrs), additional expression was seen in the cerebral hemispheres and strong expression in a salt and pepper distribution of cells in the optic tectum.

10 Cranial placodes

It is striking that C-Serrate is expressed in all the cranial placodes - the lens placode, the nasal placode, the otic placode/vesicle and the epibranchial placodes, as well as a patch of cranial ectoderm anterior to the otic placode that may correspond to the trigeminal placode (which is not well-defined morphologically).

In the lens placode, expression was already seen at stage 11, rapidly became very strong, and persisted at least to stage 21. Expression was weaker in the nasal placode and was only detected from stage 13. Again, expression was maintained at least until stage 21.

Likewise for the otic placode, expression began to be visible at stage 10 and was strong by early stage 11 (12-14 somites, 42.5 hours). Curiously, there was a "hole" in the otic expression domain - an anteroventral region of the placode in which the gene was not expressed. Subsequently, as the placode invaginates to form an otic vesicle, the strongest expression was seen at the anterolateral and posteromedial poles. Later still, as the otic vesicle becomes transformed into the membranous labyrinth of the inner ear, C-Serrate expression became restricted to the sensory patches.

The epibranchial expression was seen at stage 13/14 as strong staining in the ectoderm around the dorsal margins of the first and second branchial clefts. It was accompanied by expression of the gene in the deep part of the lining of

the clefts and in the endodermal lining of the branchial pouches, where the two epithelia abut one another.

Lastly, a large and strong but transient patch of expression was seen in the cranial ectoderm just anterior and ventral to the ear rudiment at stage 11. From its location, we suspect this to be, or to include, the region of the trigeminal placode.

Nephric mesoderm

- 10 Expression was detectable in the cells of the intermediate mesoderm from stage 10 and in older embryos (stage 17 to 21) in the developing mesonephric tubules.

Limb buds

- 15 C-Serrate mRNA was localized to a patch of mesenchyme at the distal end of the developing limb bud. This may suggest a role in limb growth.

Other sites

- 20 Expression was also seen in the tail bud, allantoic stalk, and possibly other tissues at late stages.

All major sites of C-Serrate expression lie within domains of C-Notch-1 expression

- 25 The conservation of the DSL domain and adjacent N-terminal region in C-Serrate suggests that it functions as a ligand for a receptor belonging to the Notch family. We thus expected to find sites where C-Serrate expression is accompanied by expression of a *Notch* gene. At such sites,
- 30 overlapping or contiguous expression of the two genes can be taken as an indication that cells are communicating by Serrate-Notch signalling. We have compared the expression pattern of C-Serrate, as shown by *in situ* hybridization, with that of C-Notch-1, to discover what overlaps in fact occur,
- 35 over a range of stages up to 8 days of incubation (E8). All the observed sites of C-Serrate expression indeed lay within,

or very closely adjacent to, domains of expression of C-Notch-1 (Table III).

Table III

5
COMPARISON OF C-NOTCH-1 AND
C-SERRATE EXPRESSION AT STAGE 17a

	Body region	C-Notch-1	C-Serrate
	brain and spinal cord	++ (almost everywhere)	++ (specific regions)
10	retina	++	-
	lens	+	++
	otic placode/vesicle	++	++
	epibranchial placodes	++	++
	nasal placode	++	++
15	dorsal root ganglia	+	-
	branchial mesenchyme	-	-
	branchial ectoderm	+	++ (furrows)
	branchial endoderm	+	++ (tips of pouches)
	presomitic mesoderm	++	-
	somites	++	-
20	notochord	++	-
	mesonephric kidney	++	++
	metanephric kidney	++	++
	blood vessels	++	++
	heart	+	++
25	limb bud (stage 21)	++ (AER)	++ (distal mesenchyme)

a Hamburger and Hamilton, 1951, J. Exp. Zool. 88:49-92.

30 Because of the importance of Notch and its partners in insect neurogenesis, it was of particular interest to us to see whether the homologous genes are involved in the development of the vertebrate CNS. C-Serrate is expressed in the CNS, and its pattern of expression shows a remarkable relationship to that of the Notch homologs.

35 We analyzed transverse sections through the spinal cord of a six day chicken embryo hybridized with C-Notch-1

and C-Serrate antisense RNA probes. C-Notch-1 was expressed throughout the luminal region as described previously; within this region, there were two small patches in which Serrate was strongly expressed.

5

Discussion

In *Drosophila* development, cell-cell signalling via the product of the *Notch* gene plays a cardinal role in the final cell-fate decisions that specify the detailed pattern of differentiated cell types. This signalling pathway, in which the Notch protein has been identified as a transmembrane receptor, is best known for its role in neurogenesis: loss-of-function mutations in *Notch* or any of a set of other genes required for signal transmission via Notch alter cell fates in the neuroectoderm, causing cells that should have remained epidermal to become neural instead. Notch-dependent signalling is, however, as important in non-neural as in neural tissues. It regulates choices of mode of differentiation in oogenesis, in myogenesis, in formation of the Malpighian tubules and in the gut, for example, as well as in development of the retina, the peripheral sensilla, and the central nervous system. In most of these cases the signal delivered via Notch appears to mediate lateral inhibition, a type of interaction by which a cell that becomes committed to differentiate in a particular way - for example, as a neuroblast - inhibits its immediate neighbors from doing likewise. This forces adjacent cells to behave in contrasting ways, creating a fine-grained pattern of different cell types.

There are, however, good reasons to believe that this is not the only function of signals delivered via Notch. Two direct ligands of Notch have been identified. These are the products of the Delta and Serrate genes. Both of them, like Notch itself, code for transmembrane proteins with tandem arrays of EGF-like repeats in their extracellular domain. Both the Delta and the Serrate protein have been shown to bind to Notch in a cell adhesion assay, and they

share a large region of homology at their amino-termini including a motif that is necessary and sufficient for interaction with Notch in vitro, the so-called EBD or DSL domain. Yet despite these biochemical similarities, they
5 seem to have quite different developmental functions.

Although Serrate is expressed in many sites in the fly, it is apparently required only in the humeral, wing and halteres disks. When Serrate function is lost by mutation, these structures fail to grow. Studies on the wing disc have
10 indicated that it is specifically the wing margin that depends on Serrate; when Serrate is lacking, this critical signaling region and growth centre fails to form, and when Serrate is expressed ectopically under a GAL4-UAS promoter in the ventral part of the wing disc, ectopic wing margin tissue
15 is induced, leading to ectopic outgrowths. Notch appears to be the receptor for Serrate at the wing margin, since some mutant alleles of Notch cause similar disturbances of wing margin development and allele-specific interactions are seen in the effects of the two genes.

20 Here we describe the identification and full length sequence of a homolog of the *Drosophila* gene Serrate, and identification and partial sequence of chick homologs of rat/mouse *Notch1* and *Notch2*.

Within the chick Serrate cDNA there is a single
25 open reading frame predicted to encode a large transmembrane protein with 16 EGF repeats in its extracellular domain. It has a well conserved DSL motif suggesting that it would interact directly with Notch. The intracellular domain of chick Serrate exhibits no homology to anything in the current
30 databases including the intracellular domains of *Drosophila* Delta and Serrate. It should be pointed out however that the intracellular domains of chick and human Serrate (see Section 12) are almost identical.

The spatial distributions of C-Notch-1 and
35 C-Serrate were investigated during early embryogenesis by *in situ* hybridization. C-Notch-1 and C-Serrate exhibit dynamic and complex patterns of expression including several regions

in which they are coexpressed (CNS, ear, branchial region, lens, heart, nasal placodes and mesonephros). The overlapping expression together with the finding that C-Serrate has a well conserved Notch binding domain suggests
5 that this receptor/ligand interaction has been conserved from *Drosophila* through to vertebrates.

In *Drosophila*, the Notch receptor is quite widely distributed and its ligands are found in overlapping but more restricted domains. In the chick a similar situation is
10 observed.

Fly Notch is necessary for many steps in the development of *Drosophila*; its role in lateral inhibition especially in the development of the central nervous system and peripheral sense organs being the best studied examples.
15 However, Notch is a multifunctional receptor and can interact with different signalling molecules (including Delta and Serrate) and in developmental processes that do not easily fit within the framework of lateral inhibition. While available evidence implicates Delta as the signalling
20 molecule in lateral inhibition there is no data to suggest that Serrate participates in lateral inhibition. Rather, Serrate appears to be necessary for development of the dorsal imaginal discs of the larva; that is, the humeral, haltere and wing discs. In the latter, the best studied of these
25 processes, Serrate and Notch are important for the development of the dorsoventral wing margin, a structure necessary for the organization of wing development as a whole.

That C-Serrate has a significant function can be
30 inferred from the conservation of its sequence, in particular, of its Notch-binding domain. The expression patterns reported for C-Serrate in this paper provide the following information. First, since the Serrate gene is expressed in or next to sites where C-Notch-1 is expressed
35 (possibly in conjunction with other Notch homologs), it is highly probable that C-Serrate exerts its action by binding to C-Notch-1 (or to another chick Notch homolog with a

similar expression pattern). Second, the expression in the developing kidney, the vascular system and the limb buds might reflect an involvement in inductive signalling between mesoderm and ectoderm, which plays an important part in the development of all these organs. In the limb buds, for example, C-Serrate is expressed in the distal mesoderm, and C-Notch-1 is expressed in the overlying apical ectodermal ridge, whose maintenance is known to depend on a signal from the mesoderm below. In the cranial placodes, a similar role is possible, but the evidence for inductive signalling is weaker, and C-Serrate may equally be involved in communications between cells within the placodal epithelium, for example, in regulating the specialized modes of differentiation of the placodal cells.

What might C-Serrate's function be within the curiously restricted domains of its expression in the CNS? One possibility is that it is involved in regulating the production of oligodendrocytes, which have likewise been reported to originate from narrow bands of tissue extending along the cranio-caudal axis of the neural tube.

9. ISOLATION AND CHARACTERIZATION OF HUMAN SERRATE HOMOLOGS

Clones for the human Serrate sequence were obtained as described below.

The polymerase chain reaction (PCR) was used to amplify DNA from a human placenta cDNA library. Degenerate oligonucleotide primers used in this reaction were designed based on amino-terminal regions of high homology between *Drosophila* Serrate and *Drosophila* Delta (see Fig. 5); this high homology region includes the 5' "DSL" domain, that is believed to code for the Notch-binding portion of Delta and Serrate. Two PCR products were isolated and used, one a 350 bp fragment, and one a 1.2 kb fragment. These PCR fragments were labeled with ³²P and used to screen a commercial human fetal brain cDNA library made from a 17-18 week old fetus

(previously available from Stratagene), in which the cDNAs were inserted into the *EcoRI* site of a λ -Zap vector.

The 1.2 kb fragment hybridized to a single clone out of the 10^6 clones screened. We rescued this fragment from the λ DNA by converting the isolated phage λ clone to a plasmid via the manufacturer's instructions, yielding the Serrate-homologous cDNA as an insert in the *EcoRI* site of the vector Bluescript KS- (Stratagene). This plasmid was named "pBS39" and the gene corresponding to this cDNA clone was called *Human Serrate-1* (also known as *Human Jagged-1* ("HJ1")). The isolated cDNA was 6464 nucleotides long and contained a complete open reading frame as well as 5' and 3' untranslated regions (Fig. 1). Sequencing was carried out using the Sequenase® sequencing system (U.S. Biochemical Corp.) on 5 and 6% Sequagel acrylamide sequencing gels.

The 350 bp fragment hybridized with two clones, containing cDNA inserts of approximately 1.1 and 3.1 kb in length; the plasmid constructs containing these inserts were named pBS14 and pBS15, respectively. Each clone was isolated, its respective insert rescued from the λ cDNA, and sequenced as above. The nucleotide sequence of the pBS14 insert was identical to a 1.1 kb stretch of sequence contained internally within the pBS15 cDNA insert and therefore, this clone was not characterized further. The sequence of the 3.1 kb pBS15 insert encoded a single open reading frame which spanned all but the 5' 20 nucleotides of the insert. The methionine located at the amino terminal residue of this predicted open reading was homologous to the start methionine encoded by the *Human Serrate-1* (HJ1) cDNA clone in pBS39. The gene encoding the cDNA insert of pBS15 was named *Human Serrate-2* and is also known as *Human Jagged-2* ("HJ2").

The pBS15 (HJ2) 3.1 kb insert was then labeled with ^{32}P and used to screen another human fetal brain library (from Clontech), in which cDNA generated from a 25-26 week-old fetus was cloned into the *EcoRI* site of λ gt11. This screen identified three potential positive clones. To isolate the

CDNAs, λ gt11 DNA was prepared from a liquid lysate and purified over a DEAE column. The purified DNA was then cut with *EcoRI* and the cDNA inserts were isolated and subcloned into the *EcoRI* site of Bluescript KS-. The bluescript constructs containing these cDNAs were named pBS3-15, pBS3-2, and pBS3-20. Two of these cDNA clones, pBS3-2 and pBS3-20, contained sequences that partially overlapped with pBS15 and were further characterized. pBS3-2 had a 3.2 kb insert extending from nucleotide 1210 of the pBS15 cDNA insert to just after the polyadenylation signal. The 2.6 kb insert of pBS3-20, was restriction mapped and partially sequenced to determine its 3' and 5' ends. This analysis indicated that the pBS3-20 insert had a nucleic acid sequence that was fully contained within the pBS3-2 cDNA insert and therefore, the pBS3-20 insert was not characterized further. The insert of pBS3-15 was determined to be a Bluescript vector fragment contaminant.

Alignment of the deduced amino acid sequence (SEQ ID NO:4) of the "complete" Human Serrate-2 (*HJ2*) cDNA (SEQ ID NO:3) generated on the computer with the deduced amino acid sequence of Human Serrate-1 (*HJ1*) from pBS39 (SEQ ID NO:2) revealed a gap of about 120 bases, leading to a frameshift, in the region encoded by the pBS15 (*HJ2*) insert, between the putative signal sequence and the beginning of the DSL domain (Fig. 2). The nucleotides missing in the gap of the pBS15 insert would be located between nucleotides 240 and 241 of SEQ ID NO:3. This missing region probably resulted from a cloning artifact in the construction of the Stratagene library.

Attempts to clone the 5' end of *HJ2* using anchored PCR, RACE, and Takara extended PCR techniques were unsuccessful. However, three human genomic clones potentially containing the 5' end of *HJ2* were obtained from the screening of a human genomic cosmid library in which 30 kb fragments were cloned into a unique *XhoI* site introduced into the *BamHI* site of a pWE15 vector (the unmodified vector is available from Stratagene). This cosmid library was

screened with a PCR fragment that had been amplified from the 5' end of pBS15 (HJ2) and three positive cosmid clones were isolated. Two different sets of primers were used to amplify DNA corresponding to the 5' end of pBS15 using the cosmid clones as a template, and both sets generated single bands that were subcloned, but which were determined to contain PCR artifacts. Portions of the cosmid clones are being subcloned directly without PCR, in order to obtain a portion of the cosmid clones that contains the 120 nucleotide stretch of DNA that is missing from pBS15.

The pBS39 cDNA insert, encoding the *Human Serrate-1* homolog (HJ1), has been sequenced and contains the complete coding sequence for the gene product. The nucleotide (SEQ ID NO:1) and protein (SEQ ID NO:2) sequences are shown in Figure 1. The nucleotide sequence of *Human Serrate-1* (HJ1) was translated using MacVector software (International Biotechnology Inc., New Haven, CT). The coding region consists of nucleotide numbers 371-4024 of SEQ ID NO:1. The Protean protein analysis software program from DNASTar (Madison, WI) was used to predict signal peptide and transmembrane regions (based on hydrophobicity). The signal peptide was predicted to consist of amino acids 14-29 of SEQ ID NO:2 (encoded by nucleotide numbers 410-457 of SEQ ID NO:1), whereby the amino terminus of the mature protein was predicted to start with Gly at amino acid number 30. The transmembrane domain was predicted to be amino acid numbers 1068-1089 of SEQ ID NO:2, encoded by nucleotide numbers 3572-3637 of SEQ ID NO:1. The consensus (DSL) domain, the region of homology with *Drosophila* Delta and Serrate, predicted to mediate binding with Notch (in particular, Notch ELR 11 and 12), spans amino acids 185-229 of SEQ ID NO:2, encoded by nucleotide numbers 923-1057 of SEQ ID NO:1. Epidermal growth factor-like (ELR) repeats in the amino acid sequence were identified by eye; 15 (full-length) ELRs were identified and 3 partial ELRs as follows:

ELR 1: amino acid numbers 234 - 264

ELR 2: amino acid numbers 265 - 299

- 5 ELR 3: amino acid numbers 300 - 339
 ELR 4: amino acid numbers 340 - 377
 ELR 5: amino acid numbers 378 - 415
 ELR 6: amino acid numbers 416 - 453
 ELR 7: amino acid numbers 454 - 490
 ELR 8: amino acid numbers 491 - 528
 ELR 9: amino acid numbers 529 - 566
 Partial ELR: amino acid numbers 567 - 598
 Partial ELR: amino acid numbers 599 - 632
- 10 ELR 10: amino acid numbers 633 - 670
 ELR 11: amino acid numbers 671 - 708
 ELR 12: amino acid numbers 709 - 747
 ELR 13: amino acid numbers 748 - 785
 ELR 14: amino acid numbers 786 - 823
- 15 ELR 15: amino acid numbers 824 - 862
 Partial ELR: amino acid numbers 863 - 879
 Partial ELR: amino acid numbers 880 - 896
- The total ELR domain is thus amino acid numbers 234 - 896
 (encoded by nucleotide numbers 1070 - 3058 of SEQ ID NO:1).
- 20 The extracellular domain is thus predicted to be amino acid
 numbers 1 - 1067 of SEQ ID NO:2, encoded by nucleotide
 numbers 371 - 3571 of SEQ ID NO:1 (amino acid numbers
 30 - 1067 in the mature protein; encoded by nucleotides
 number 458 - 3571 of SEQ ID NO:1). The intracellular
- 25 (cytoplasmic) domain is thus predicted to be amino acid
 numbers 1090 - 1218 of SEQ ID NO:2, encoded by nucleotide
 numbers 3638 - 4024 of SEQ ID NO:1.

The expression of *HJ1* in certain human tissues was
 established by probing a Clontech Human Multiple Tissue

30 Northern blot with radio-labeled pBS39. The probe hybridized
 to a single band of about 6.6 kb, and was expressed in all of
 the tissue assayed, which included, heart, brain, placenta,
 lung, skeletal muscle, pancreas, liver and kidney. The
 observation that *HJ1* was expressed in adult skeletal and

35 heart muscle was particularly interesting, because adult
 muscle fibers are completely surrounded by a lamina of
 extracellular matrix, and it is unlikely, therefore, that the

role of HJ1 in these cells is in direct cell-cell communication.

The "complete" (containing an internal deletion) *Human Serrate-2 (HJ2)* cDNA nucleotide sequence (SEQ ID NO:3) and amino acid sequence (SEQ ID NO:4) generated on the computer are shown in Figure 2. The nucleotide sequence translated using MacVector software (International Biotechnology Inc., New Haven, CT). The coding region consists of nucleotides number 332 - 4102 of SEQ ID NO:3.

10 The Protean protein analysis software program from DNASTar (Madison, WI) was used to predict signal peptide and transmembrane regions (based on hydrophobicity). The transmembrane domain was predicted to be amino acid numbers 912-933 of SEQ ID NO:4, encoded by nucleotides numbers 3065-3130 of SEQ ID NO:3. The consensus (DSL) domain, the region of homology with *Drosophila* Delta and Serrate, predicted to mediate binding with Notch (in particular, Notch ELR 11 and 12), spans amino acids 26-70 of SEQ ID NO:4, encoded by nucleotide numbers 407 - 541 of SEQ ID NO:3.

20 Epidermal growth factor-like (ELR) repeats in the amino acid sequence were identified by eye; 15 (full-length) ELRs were identified and 3 partial ELRs as follows:

- ELR 1: amino acid numbers 75 - 105
- ELR 2: amino acid numbers 106 - 140
- 25 ELR 3: amino acid numbers 141 - 180
- ELR 4: amino acid numbers 181 - 218
- ELR 5: amino acid numbers 219 - 256
- ELR 6: amino acid numbers 257 - 294
- ELR 7: amino acid numbers 295 - 331
- 30 ELR 8: amino acid numbers 332 - 369
- ELR 9: amino acid numbers 370 - 407
- Partial ELR: amino acid numbers 408 - 435
- Partial ELR: amino acid numbers 436 - 469
- ELR 10: amino acid numbers 470 - 507
- 35 ELR 11: amino acid numbers 508 - 545
- ELR 12: amino acid numbers 546 - 584
- ELR 13: amino acid numbers 585 - 622

ELR 14: amino acid numbers 623 - 660

ELR 15: amino acid numbers 664 - 701

Partial ELR: amino acid numbers 702 - 718

Partial ELR: amino acid numbers 719 - 735

- 5 The total ELR domain is thus amino acid numbers 75 - 735
(encoded by nucleotides number 554 - 2536 of SEQ ID NO:3).
The extracellular domain is thus predicted to be amino acid
numbers 1 - 912 of SEQ ID NO:4, encoded by nucleotides number
332 - 3064 of SEQ ID NO:3. The intracellular (cytoplasmic)
10 domain is thus predicted to be amino acid numbers 934 - 1257
of SEQ ID NO:4, encoded by nucleotide numbers 3131 - 4102 of
SEQ ID NO:3.

- Like *Human Serrate-1 (HJ1)*, the "complete" (with an
internal deletion) *Human Serrate-2 (HJ2)* cDNA (SEQ ID NO:3)
15 generated on the computer encodes a protein containing 16
complete and 2 interrupted EGF repeats as well as the
diagnostic cryptic EGF repeat known as the DSL domain, which
has been found only in putative Notch ligands. The open
reading frame of the computer generated "complete" *Human*
20 *Serrate-2 (HJ2)* is about 1400 amino acids long, approximately
182 amino acids longer than the carboxy terminus of HJ1 and
the rat *Serrate* homologue *Jagged*. While there is significant
homology between the complete HJ2 and HJ1 in the amino
terminal portion of the protein, this homology is lost just
25 before the putative transmembrane domain at about amino acid
number 1029 of HJ1. This result is particularly interesting
because the presence of a long COOH-terminal tail implies the
possibility of some additional function or regulation of HJ2.

- The "complete" (with an internal deletion) *Human*
30 *Serrate-2 (HJ2)* cDNA (SEQ ID NO:3) sequence can be
constructed by taking advantage of the unique restriction
sites for *AccI*, *DraIII*, or *BamHI* present in the sequence
overlap of pBS15 and pBS3-2, and which enzymes cleave the
pBS15 insert at nucleotides 1431, 2648, and 2802,
35 respectively.

The expression of *HJ2* in certain human tissues was established by probing a Clontech Human Multiple Tissue Northern blot with radio-labeled clone pBS15. This probe hybridized to a single band of about 5.2 kb and was expressed in heart, brain, placenta, lung, skeletal muscle, and pancreas, but was absent or nearly undetectable in liver and kidney. As in the case of *HJ1* expression discussed supra, the observation that the pBS15 insert component of *HJ2* was expressed in adult skeletal and heart muscle was particularly interesting, because adult muscle fibers are completely surrounded by a lamina of extracellular matrix, and it is unlikely, therefore, that the role of *HJ2* in these cells is in direct cell-cell communication.

Expression constructs are made using the isolated clone(s). The clone is excised from its vector as an *EcoRI* restriction fragment(s) and subcloned into the *EcoRI* restriction site of an expression vector. This allows for the expression of the Human Serrate protein product from the subclone in the correct reading frame. Using this methodology, expression constructs in which the *HJ1* cDNA insert of pBS39 was cloned into an expression vector for expression under the control of a cytomegalovirus promoter have been generated and *HJ1* has been expressed in both 3T3 and HAKAT human keratinocyte cell lines.

25

10. DEPOSIT OF MICROORGANISMS

Plasmid pBS39, containing an *EcoRI* fragment encoding full-length Human Serrate-1 (*HJ1*), was deposited on February 28, 1995 with the American Type Culture Collection, 1201 Parklawn Drive, Rockville, Maryland 20852, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures, and assigned Accession No. 97068.

Plasmid pBS15, containing a 3.1 kb *EcoRI* fragment encoding the amino terminus of Human Serrate-2 (*HJ2*), cloned into the *EcoRI* site of Bluescript KS-, was deposited on March 5, 1996 with the American Type Culture Collection, 1201

Parklawn Drive, Rockville, Maryland 20852, under the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures, and assigned Accession No. _____.

5 Plasmid pBS3-2 containing an 3.2 kb *EcoRI* fragment encoding the carboxy terminus of Human Serrate-2 (HJ2), cloned into the *EcoRI* site of Bluescript KS-, was deposited on March 5, 1996 with the American Type Culture Collection, 1201 Parklawn Drive, Rockville, Maryland 20852, under the
10 provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures, and assigned Accession No. _____.

The present invention is not to be limited in scope
15 by the microorganisms deposited or the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are
20 intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

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30

35

SEQUENCE LISTING

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 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: To Be Assigned
 - (B) FILING DATE: On Even Date Herewith
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
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 - (B) REGISTRATION NUMBER: 18,872
 - (C) REFERENCE/DOCKET NUMBER: 7326-037-228
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (212) 790-9090
 - (B) TELEFAX: (212) 869-9741/8864
 - (C) TELEX: 66141 PENNIE

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6464 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: double
 - (D) TOPOLOGY: unknown
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 371..4027